FIELD-EVOLVED RESISTANCE TO CRY3Bb1 CORN BY THE WESTERN CORN ROOTWORM: INVESTIGATIONS OF RESISTANCE, EMERGENCE, NODE-INJURY RATINGS, AND FITNESS

BY

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THESIS

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ABSTRACT

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is an economically relevant pest of corn, *Zea mays* L. In 2003, the United States Environmental Protection Agency (US EPA) approved the use of transgenic Bt (*Bacillus thuringiensis* Berliner) corn hybrids to manage the larvae of this billion dollar pest. Bt hybrids express various toxic Cry proteins (i.e., Cry3Bb1, Cry34Ab1/Cry35Ab1, mCry3A, eCry3.1Ab) which have proven to be valuable tools for protecting corn roots from western corn rootworm larval feeding. However, the western corn rootworm has repeatedly developed resistance to management techniques such as crop rotation (by ovipositing in non-corn crops) and some conventional insecticides, which supports the expectation that evolution of resistance by the western corn rootworm is the main threat to the continued success of Bt corn.

The first documented case of field-evolved resistance to Bt, by western corn rootworms, occurred in 2009 in Iowa to the Cry3Bb1 protein (Gassmann et al. 2011). Field-evolved resistance occurs as a genetically-based decrease in susceptibility of a population to a toxin caused by repeated exposure, and subsequent selection pressure, of the population to the toxin in the field. The potential for resistance has been compounded by several factors including the fact that the Cry3Bb1 toxin is expressed at a low dose rather than a high dose. Low dose expression increases the survival of heterozygous insects, those individuals with resistant and susceptible alleles. A lack of grower compliance with refuge requirements also increases selection pressure for resistance development. The initial resistance allele frequency is also higher than what would be expected for adequate resistance prevention provided by current refuge standards. Furthermore, several researchers have described fitness costs, of western corn rootworms associated with resistance to Cry3Bb1, as being negligible.

The central goal of this two-year, multi-site field experiment was to determine if field-evolved resistance to the Cry3Bb1 toxin by the western corn rootworm had occurred in Illinois and then evaluate

the suspected resistant population. Plant based bioassays were conducted by Dr. Aaron Gassmann's laboratory at Iowa State University and confirmed Cry3Bb1 resistance of the western corn rootworms at our study located in LaSalle and Whiteside Counties. Field experiments were established to evaluate node injury ratings, adult emergence, and to test for potential fitness costs associated with Bt resistance. Field studies included treatments with commercially available Bt hybrids and their corresponding near isolines.

Experiments were conducted in producers' fields where Cry3Bb1 performance problems had been observed in previous years. During my research in LaSalle and Whiteside Counties in 2013, I observed similar nodal root injury between corn hybrids expressing either the Cry3Bb1 or mCry3A proteins compared to their respective untreated check, which lacked rootworm Bt proteins. In contrast, I observed reduced nodal root injury to corn hybrids expressing the Cry34/35Ab1 protein compared to their respective untreated checks in 2013. I also observed reduced mean male cumulative emergence from corn pyramided hybrids expressing the Cry34/35Ab1 protein when compared to their respective untreated checks at the LaSalle and Whiteside County sites. Although head capsule widths and adult dry weights fluctuated among treatments at some sites, Bt corn did not have a consistent effect on these fitness measurements which suggests a lack of fitness costs associated with Cry3Bb1 resistance.

With the global importance of transgenic crop technology and its use as a tool for successfully managing insect pests, it is important to prolong its usefulness. It is clear that further investigation is required to identify Bt product failures, and improve decisions regarding insect resistance management to ensure the durability and longevity of each Bt product.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is an economically significant pest of corn, *Zea mays* (L.), in North America and more recently in Europe. This major corn pest originated in Central America, in what is today Guatemala (Melhus et al. 1954). The western corn rootworm has been described in the United States as early as 1867 (Chiang 1973). Gillette (1912) described sweet corn damage in Colorado caused by the western corn rootworm in 1909. Gray et al. (2009) described the ensuing range expansion across the United States and into Europe as the corn monoculture cropping system took hold. There have been five independent introductions of this pest into Europe which has resulted in its damaging spread throughout the continent (Miller et al. 2005).

In the United States, the combination of annual yield losses and management costs of the corn rootworm complex, which includes the western corn rootworm, the northern corn rootworm (*Diabrotica barberi* Smith & Lawrence), and the southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber), have been estimated to exceed $1 billion annually (Metcalf 1986). In Dun et al. (2010), the current cost is estimated to be significantly larger due to factors including inflation, an increase in market price of corn, and the recent surge in seed costs associated with genetically engineered traits. Understanding the biology and life history of the western corn rootworm as well as host-plant relationships is essential to successfully managing this economically important pest of corn.

BIOLOGY

Eggs

Female western corn rootworms oviposit their eggs in the soil late in the summer (Ball 1957; Hein and Tollefson 1985). Oviposition primarily occurs in corn fields (Spencer and Levine 2008). Approximately 23% of eggs are laid in the upper 2 inches (5.08 cm) of soil, 58% in the upper 4 inches (10.16 cm), and the remaining 20% below the 6-inch (15.24 cm) level (Ball 1957).

The western corn rootworm spends the majority of its life overwintering in the egg stage where it commonly remains in diapause throughout the winter (Chiang 1973). Cold temperatures induce chill-quiescence, which prevents eggs from developing prior to optimal spring conditions. Synchronization of egg development with corn production through egg diapause ensures rootworm survival in a fluctuating seasonal climate with harsh environmental extremes (Coats et al. 1986). These rough environmental conditions are discussed in Toepfer and Kuhlmann (2006) where it was estimated that 46% of diapausing eggs die while overwintering according to life table experiments. Diapause can be broken artificially by chilling the eggs for prescribed periods at various temperatures; however, mortality due to premature diapause termination under field conditions is rare (Gustin 1981).

Larvae

Western corn rootworm larvae hatch in the spring and are generally monophagous feeders of corn roots (Branson and Ortman 1967; Levine and Oloumi-Sadeghi 1991; Krysan 1993; Moeser et al. 2005). However, the western corn rootworm can complete development on some grass species (Clark and Hibbard 2004; Wilson and Hibbard 2004). Oyediran et al. (2004) determined in greenhouse assays that 14 of 23 plant species support adult emergence and Oyediran et al. (2008) found that rootworm larvae can also complete development and emerge on 6 of 27 prairie grass species. Before pupation occurs, larvae undergo three instars near the soil surface (Spike and Tollefson 1991; Hammack et al. 2003). Larvae are highly attracted to the root volatile carbon dioxide, and use these volatiles to

recognize feeding sites (Strnad et al. 1986; Bernklau and Bjostad 1998). Larvae typically feed on the youngest roots (Spencer et al. 2009). Hibbard et al. (2004) determined that initial larval establishment is not dependent on the density of corn but later movement is density dependent. Larvae move from highly damaged and infested plants to plants that have less damage and feeding (Hibbard et al. 2003). Larvae also move towards younger roots as the plant develops (Strnad and Bergman 1987). The failure of larvae to locate corn roots after hatching results in high larval mortality (Branson 1989; Oloumi-Sadeghi and Levine 1989; Toepfer and Kuhlmann 2006).

The western corn rootworm is most economically destructive in its larval stage. Larval feeding causes root injury and reduced nutrient and water uptake (Kahler et al. 1985; Spike and Tollefson 1991), which can reduce a plant's photosynthesis rate (Godfrey et al. 1993; Riedell and Reese 1999; Urías-López et al. 2000). Moellenbeck et al. (2001) observed that larval feeding weakens root structure, increases crop vulnerability to disease and drought, and negatively effects stability. Tinsley et al. (2012), described that one node of corn roots lost from feeding is equivalent to a 15% yield loss. Larval feeding on corn roots can result in lodging which can negatively affect yield by complicating mechanical harvest (Riedell 1990). Larvae pupate and eclose into adults in late June or early July (Spencer et al. 2009).

Adults

Western corn rootworms are univoltine and primarily consume corn tissues (Chiang 1973). These corn tissues include silks, kernels, and tassels (Levine and Oloumi-Sadeghi 1991; Moeser and Vidal 2005; Gray et al. 2009). Damage can be caused by adults when beetles prevent pollination by clipping silks; however, adults are often not considered economically important (Clark and Hibbard 2004). Beetle dispersal by flight is greatest before and after flowering intervals as a response to changes in habitat quality (Naranjo 1994). Adult rootworms are typically present from June until there is frost in the fall (Spencer et al. 2009).

Mean male emergence typically occurs 5-7 days before mean female emergence (Branson 1987, Murphy et al. 2010; Quiring and Timmins 1990). While western corn rootworms are protandrous, over 98% of male emergence coincides with female emergence (Quiring and Timmins 1990). Male western corn rootworms typically need several days to sexually mature (Spencer et al. 2009). Males may mate multiple times while females typically mate once (Branson et al. 1977). Females generally mate quickly, with 96% mating after only 24 hours (Quiring and Timmins 1990). Hammack (1995) described that female western corn rootworms display a postural display or call posture. Females then release a complex sex pheromone which consists of 8-methyl-2-decanol propanoate (Guss et al. 1982). Females start to oviposit in 6-10 days and produce eggs 4-5 weeks after mating (Sherwood and Levine 1993). Females live an average of 78.2 days and have a mean fecundity of 1087 eggs laid in 13.5 clutches over a reproductive period of 76.4 days (Hill 1975).

After western corn rootworms mate, females feed for several days then begin to disperse from their natal field before oviposition (Isard et al. 2004).Females prefer to oviposit within moist soil substrates (Gustin 1979), and will also enter drought cracks and oviposit at a variety of soil depths (Kirk 1979).

MANAGEMENT TECHNIQUES

Measuring Injury and Damage

The central management philosophy of western corn rootworm control focuses on the reduction of larval feeding on crop roots. Several scales have been used historically to measure root injury caused by the western corn rootworm. Hills and Peters (1971) first developed a 1-6 injury categorical scale. Next, a 1-9 scale was developed (Musick and Suttle 1972). Oleson et al. (2005) described the now commonly used node-injury scale which is based on a 0-3 quantitative scale. Tinsley et al. (2012) estimated that a 15% reduction in yield occurs for one node, or circle of roots, consumed by western corn rootworm larvae. The larval economic threshold has been determined to be 10 larvae per

plant (Peters 1963). The economic threshold for adults has been set at a whole plant observation of 1.62 adults per plant in continuous corn and 0.71 adults per plant in first year corn (Godfrey and Turpin 1983). Additionally, if greater than 6 adults are collected per sticky trap per day in continuous corn, the economic threshold has been reached (Hein and Tollefson 1985). If greater than 4.7 adults are collected per trap per day in soybeans for rotated corn, the economic threshold has also been reached (O'Neal et al. 2001).

Chemical Control

Insecticides have been used to control the western corn rootworm to preserve crop yields since the pest was first described as a crop hazard in the United States. Chemical control was used in the 1940's with benzene hexachloride applications on the soil surface (Hill et al. 1948). Chemically controlling adult western corn rootworms typically means reducing the amount of eggs deposited by female beetles in the field which in turn reduces the amount of larvae present the following year (Levine and Oloumi-Sadeghi 1991; Meinke et al. 1998; Clark and Hibbard 2004). Most insecticides are used as corn seed treatments or are applied as soil insecticides at the time of planting (Levine and Oloumi-Sadeghi 1991). Additionally, foliar applied broad spectrum insecticides can be applied to grower fields to decrease the number of eggs laid in an attempt to reduce the following year's larval damage (Levine and Oloumi-Sadeghi 1991).

The western corn rootworm has evolved resistance to several insecticides (Ball and Weekman 1962; Meinke et al. 1998; Wright et al. 2000; Siegfried et al. 2004). However, resistance to granular insecticides which are applied in the furrow or in a narrow band over corn rows has not yet occurred. This is most likely due to the functional refuge provided between the bands of insecticides that are applied at planting.

Crop Rotation

Crop rotation has been used to control the western corn rootworm for over a century (Krysan 1993; Spencer and Levine 2008). Adult western corn rootworms have a high ovipositional fidelity to corn which makes crop rotation a historically effective solution for this pest as larvae do not survive well on other crops (Levine and Oloumi-Sadeghi 1991, O'Neal et al. 1999, Clark and Hibbard 2004, Spencer et al. 2009). Western corn rootworm larvae will not survive in soybean fields because they lack a suitable host (Short and Luedtke 1970, Hibbard et al. 2004). Bullock (1992) determined that a 2 year rotation of corn and soybeans increases yield by 5-20%. Using crop rotation as a management strategy eliminates the need for a Bt hybrid for western corn rootworm in many areas of the Corn Belt. The use of a non-Bt hybrid would remove the trait fees that are commonly associated with Bt corn. As a management tactic, crop rotation reduces chemical control which improves the overall health of the environment (Pimentel et al. 1993).

Crop rotation remained an effective for western corn rootworm control since it was first recommended as a management tactic in the early 1900s (Gillette 1912). Severe root injury to first-year corn was observed in Illinois (Piper City, Ford County) in 1987 (Levine and Oloumi-Sadeghi 1996). According to Gray et al. (2009), the current range of rotation-resistant western corn rootworm includes the northern two-thirds of Illinois and Indiana, as well as portions of eastern Missouri and Iowa, Southern Wisconsin and Michigan; and western Ohio. Rotation-resistant western corn rootworms can also oviposit in oats, alfalfa, and winter wheat (Rondon and Gray 2004; Schroeder et al. 2005). Rotation resistance is most commonly associated with soybean because the corn soybean rotation occurs at the highest incidence within the Corn Belt (Onstad et al. 2003). Rotation-resistant western corn rootworms, also called variant western corn rootworms, have behavioral adaptations that influence their oviposition in soybean fields (Levine et al. 2002). The underlying mechanism of rotation-resistance has been attributed to a lack of ovipositional fidelity to corn (Rondon and Gray 2004). Pierce and Gray (2006)

suggested that rotation-resistance was facilitated by decades of rigid annual crop rotation of corn and soybean. Curzi et al. (2012) discovered that the rotation-resistant western corn rootworm had 3 to 4 times more of a key digestive enzyme (cathepsin L-like protease) enabling them to feed on soybean foliage for longer periods of time. As a result, these western corn rootworms spent more time in soybean fields laying eggs (Curzi et al. 2012).

Host Plant Resistance

Host plant resistance is a common management strategy commonly used to control crop pests. Painter (1951) divided host plant resistance into three categories including (1) nonpreference (2) antibiosis, and (3) tolerance. Nonpreference was later renamed antixenosis (Kogen and Ortman 1978). The search for western corn rootworm host plant resistance began in the 1930s (Bigger et al. 1938). Root injury was determined to be the best method of evaluating the efficacy of host plant resistance (Knutson et al. 1999). Larger root size and secondary root regrowth positively correlates with a crop's yield potential (Tollefson 2007). Corn hybrids produced in the 1980's commonly exhibited tolerance to the western corn rootworm (Riedell and Evenson 1993). Antibiosis, in the form of hydroxamic acid presence in root tissues, was found to be a potential form of host plant resistance (Assabgui et al. 1995a; 1995b). Survival of western corn rootworms was found to be inversely related to the amount of hydroxamic acid in roots (Assabgui et al. 1995 a). Khishen et al. (2009) described two non-genetically engineered corn lines that showed antibiosis resistance.

Bt Crops

The arrival of genetic engineering of plants initiated the integration of novel genes into crop genomes. *Bacillus thuringiensis* (Berliner) (Cyanobacteria: Bacillales) is a naturally occurring, gram-positive soil borne bacterium that forms spores during the stationary phase (low or no growth) which produce a protein-based crystal which makes it toxic to target arthropods. The genes that code for the production of these toxins have been integrated into crop genomes, and are called Bt crops (Ostlie et al.

1997; Schnepf et al. 1998). The crystalline protoxin produced in crop tissues must be consumed by a susceptible insect. The protoxin is then solubilized in the insect's alkaline midgut and activated by proteases that cleave out the active protein. The active protein then binds to the midgut epithelial lining via cadherin receptors. Once the protein binds to the cell membrane, it inserts itself into the cell membrane forming a pore in the cell. The pore allows ions and cellular contents to escape which results in cell lysis. When enough of the midgut cells lyse the insect will experience sepsis and die (Gill et al. 1992; Knowles 1994; Tabashnik and Carrière 2008; Bravo et al. 2011).

The first transgenic Bt crop was cotton and was commercialized in 1996 to control Lepidoptera pests of cotton(Gould 1998). Since then, many Bt control technologies have been produced that focus on controlling insect larvae from the orders Lepidoptera (caterpillars), Diptera (mosquitos, flies), Coleoptera (beetles), and Hemiptera (lygus bug) (Tabashnik and Carrière 2008; Baum et al. 2012). Certain Bt crystal proteins have been reported as having activity against non-insect species such as nematodes. Bt-based insecticides have the advantage of being selectively poisonous to specific target insect species without exhibiting any mammalian toxicity. The use of transgenic plants initially reduced insecticide applications and personal exposure to insecticides. These rootworm-Bt hybrids have reduced the application of insecticides that are harmful to the environment as far less foliar or soil applied broad spectrum insecticides are needed (Rice 2004). The Environmental Protection Agency (EPA) has mandated that all registrants submit an insect resistance management plan before the registration of any Bt crop. Resistance management, or maintaining pest susceptibility to control tactics, can extend the lifetime of corn rootworm products.

Drieg et al. (1983) observed *Bacillus thuringiensis* pathotypes that were active against Coleoptera larvae. Nearly a decade later, novel three domain Cry crystal proteins with activity towards coleopteran insect larvae were discovered (Lambert et al. 1992). Further screening found Cry34 and Cry35-type crystal proteins that showed activity against western corn rootworm larvae (Ellis et al. 2002).

In 2003, the first transgenic rootworm Bt hybrid, YieldGard Rootworm (Event MON-ØØ863-5, Monsanto Co., St. Louis, MO), was approved by the US EPA (Vaughn et al. 2005). YieldGard RW produces the insecticidal toxin Cry3Bb1. This Bt toxin is expressed in below and above ground tissues (Vaughn et al. 2005) and its expression in root tissues is intended to kill feeding western corn rootworm larvae (Vaughn et al. 2005). Rice (2004) observed that the Cry3Bb1 toxin expressed in roots worked as well or better than soil-applied insecticides. Similarly, Clark et al. (2006) reported that neonates decreased feeding on roots expressing the Cry3Bb1 toxin compared with susceptible cultivars.

Herculex Rootworm (Event DAS-59122-7, DuPont Pioneer, Johnston, IA, and Dow AgroSciences LLC, Indianapolis, IN) was described as active against the western corn rootworm by Baum et al. (2004) and was approved for commercial use as a binary toxin in 2006 by the US EPA (2005). This was followed by the 2007 approval of Agrisure Rootworm (Event SYN-IR6Ø4-5, Syngenta, Basel, Switzerland) (Head and Ward 2009). Walters et al. (2008) described the mCry3A toxin as active against western corn rootworm larvae. Further developments included the first pyramided product for rootworm control expressing both Cry3Bb1 and Cry34/35Ab1, under the trade name SmartStax, in 2009; and the pyramid expressing both mCry3A and Cry34/35Ab1, under the trade name Agrisure 3122 in 2012 (US EPA 2009; US EPA 2011). Bt hybrids that are pyramids express two or more Cry toxins to control a single pest. Additionally, Syngenta's Agrisure Durricade, which includes the novel toxin eCry3.1Ab (event 5307) in a pyramid with the mCry3A toxin, was deregulated for the 2014 growing season. An added benefit provided by pyramided Bt hybrids is that resistance evolution is generally delayed unless soil insecticide is used in the refuge (Onstad and Meinke 2010).These Bt toxins provide root protection that is as good, or better than is possible with a soil-applied insecticide, but significant damage can still occur at high levels of western corn rootworm pressure (Gray et al. 2007) or if the western corn rootworm population is resistant to the corresponding Bt protein (Gassmann et al. 2012).

Later western corn rootworm instars are less susceptible to Cry3Bb1, Cry34/35Ab1, and mCry3A (deployed as a single toxin) than neonates, and have the potential to survive on these single toxin hybrids. Root growing points have a greater concentration of soluble Bt toxins compared with older root tissues (Lefko et al. 2008; Meissle et al. 2009). Hibbard et al. (2005) reported that neonate and later instars preferred near-isogenic corn roots to Cry3Bb1 expressing roots given the choice. Clark et al. (2006) similarly described that western corn rootworm larvae may be able to detect small differences in the expression of the Cry3Bb1 protein in the root system and adapt their feeding behavior to avoid the more-toxic root parts. However, Petzold-Maxwell et al. (2012) determined that western corn rootworm larvae from both Cry3Bb1 resistant and Cry3Bb1 susceptible strains did not exhibit behavior to reduce exposure to the Cry3Bb1 protein when given a choice between Bt and non-Bt corn.

Insect Resistance Management for Bt

The long-term success of the transgenic approach to rootworm control will depend on appropriate and effective insect resistance strategies. Resistance evolution has been a threat to the efficacy of Bt particularly due to the western corn rootworm's history of overcoming management tactics. The US EPA has mandated that growers implement a refuge strategy in order to maintain the efficacy of Bt crops. Planting non-Bt host plants along with Bt crops is intended to slow resistance development (Gould 1998). The purpose of planting a refuge is to promote the mating of the rare resistant beetles with abundant susceptible beetles from refuge plants without Bt toxins. (Carrière and Tabashnik 2001). If inheritance of resistance is recessive, the offspring produced by mating resistant with susceptible beetles would be killed by Bt crops, which would slow resistance development (Tabashnik et al. 2008). This approach is referred to as the high dose/refuge strategy and has been described as the preferred resistance management strategy by the US EPA (US EPA 1998; Glaser and Matten 2003). However, the effectiveness of this strategy depends on the assumption that very few pests survive after exposure to Bt expressing tissues.

The historic success of Bt traits targeting lepidopteran pests helped to promote the implementation of the high dose/refuge strategy for coleopteran targeting Bt products. Current commercialized Bt single traits targeting rootworm have been considered low dose, low to moderate dose and less than high dose. Bt refuge requirements for rootworm control were initially modeled after the refuge methodology of Bt products that target Lepidoptera pests. In this high dose refuge strategy, highly toxic, high-dose Bt varieties are planted as monocultures, and non-Bt varieties are planted in either separate fields, as blocks or strips within the same fields, in so-called structured refuges, in which there is less selection for resistance (Gould and Tabashnik 1998). Improved efficacy of the high dose-refuge strategy has been shown when resistance is recessive, and monogenic (Tabashnik and Croft 1982). Bt varieties that are high dose and cause significant (>99%) mortality may work well with structured refuges for some lepidopteran pests (Gould 1998).

Resistance evolution is affected by the availability of susceptible refuge insects, type of resistance inheritance, and potential fitness cost associated with Bt resistance. To help prevent the development of resistance, non-Bt plants are planted as a refuge (Gould 1998). Homozygous resistant beetles that mate with homozygous susceptible beetles produce progeny that are heterozygous yet still susceptible to the Bt toxin. This sustains the efficacy for the Bt control product for a longer duration (Tabashnik and Carrière 2008). The delay in resistance is greatest when resistance is completely recessive (Tabashnik et al. 2004). Planting a larger amount of refuge also coincides with a greater delay in resistance (Carrière and Tabashnik 2001). Single toxin Bt corn targeting the western corn rootworm requires a 20% refuge to be planted adjacent to or within the field containing the Bt hybrid (DiFonzo and Cullen 2012). Corn hybrids that express multiple rootworm Bt toxins require a smaller refuge (5%) (DiFonzo and Cullen 2012). The use of pyramided Bt hybrids help increase the mortality of heterozygous larvae which is important because Bt is a low dose event (Ives et al. 2011). Many scientists have suggested that refuge standards should be increased to 50% for hybrids expressing a single Cry toxin and

20% for hybrids expressing two Cry toxins because Bt corn is not high dose for the western corn rootworm (Tabashnik and Gould 2012). Prior to the commercialization of Cry3Bb1, the majority of a 2002 Scientific Advisory Panel recommended a large refuge (at least 50%) to the U.S. Environmental Protection Agency as a resistance management plan for the western corn rootworm (Tabashnik and Gould 2012).

Fitness costs accompanying Bt resistance occur, when in the absence of Bt toxins, fitness is lower for resistant insects than for susceptible insects (Gassmann et al. 2009). Additionally, fitness costs delay resistance by reducing the frequency of resistant alleles (Carrière and Tabashnik 2001; Pittendrigh et al. 2004; Gassmann et al. 2009). This is accomplished by selecting against resistant genotypes, thereby increasing the effectiveness of refuges for delaying resistance (Gould 1998; Carrière and Tabashnik 2001; Gassmann et al. 2009). Fitness costs may affect survival, egg viability, fecundity, developmental rate, adult longevity, and overwintering survival (Carrière et al. 2001; Janmaat and Myers 2005). Gassmann et al. (2009) investigated 77 studies which included 18 species and found fitness costs were detected in 62% of experiments. Most fitness costs were recessive, but non-recessive costs can select more strongly against resistance.

WCR Resistance to Bt

Wide-spread planting of Bt corn has placed a large amount of selection pressure for resistance evolution in a field setting. Grower adoption of Bt corn has grown considerably since its inception (Onstad et al. 2011). In 2013, 76% of the U.S. and 82% of the Illinois corn crop was planted with Bt varieties (USDA 2014). The large geographical area of Bt crops targeting the western corn rootworm creates significant selection pressure on this pest to overcome Bt as a control mechanism (head and Greenplate 2012). Tabashnik et al. (2014) describes resistance as a genetically based decrease in susceptibility to a pesticide. Field-evolved resistance is equivalent to field-selected resistance and can be described as a genetically based decrease in susceptibility to a pesticide in a population caused by

exposure to the pesticide in the field (Tabshnik et al. 2014). The severity of field-evolved resistance can vary greatly so practice resistance, also called field resistance, has been coined in Tabashnik et al. (2014) as field-evolved resistance that reduces pesticide efficacy and has practical consequences for pest control.

The concern of widespread use of Bt toxins on continuous corn has been compounded by a lack of adequate grower compliance with refuge requirements. Gray (2011a) surveyed farmers in Illinois to determine their refuge practices. In this study, compliance was found to range from 75 to 82% (Gray 2011a). In 2010 Minnesota growers were also surveyed and reported very low levels of compliance (only 2%) because the refuge was not in wide enough strips or blocks (Andow et al.2010). The increase in grower adoption of seed blend refuge has helped alleviate the lack of grower compliance associated with the use of a structured refuge. However, research is still needed to determine if the reduced refuge sizes used for seed blends are adequate from a resistance management standpoint.

A resistant western corn rootworm colony was selected for resistance to the Cry3Bb1 protein in a laboratory after as few as three generations (Meihls et al. 2008). Larval survival of the resistant strain on Bt-corn was equivalent to survival on the near-isogenic line. Similarly after three generations, the LC50 (toxin concentration causing 50% mortality) of the colony continuously exposed to Bt-corn was approximately 22-fold greater than that of the colony reared on the near-isogenic corn. Several Iowa grower fields developed resistant western corn rootworms to Cry3Bb1 corn after three to seven generations of selection (Gassmann et al. 2011, 2012). Gassmann et al. (2011) reported severe root injury and lodging in fields with hybrids expressing the Cry3Bb1 toxin. Resistance to the Cry3Bb1 protein persisted after a lack of Cry3Bb1 hybrids for 2 years with eleven times greater survival for Cry3Bb1 resistant larvae. This suggests the resistance mechanism most likely persists in the western corn rootworm population (Gassmann et al. 2012). There have been multiple subsequent occurrences of

Cry3Bb1 performance failures in: Illinois, Iowa, Minnesota, Nebraska, and South Dakota (Gray 2011a,b,c; Gray 2012; US EPA 2011; Porter et al. 2012).

Gassmann et al. (2011) developed plant based bioassays for detecting resistance. These bioassays revealed three times greater survival of larvae, the offspring of adults coming from grower fields, that in 2009 had severe western corn rootworm feeding damage to Cry3Bb1-expressing hybrids compared with larvae reared from adults collected from fields that did not report damage (control fields) (Gassmann et al. 2011). Oswald et al. 2011 described western corn rootworm resistant larvae survival to be four times greater than Bt-susceptible larvae to the Cry3Bb1 protein.

Gassmann et al. (2011, 2012) found a significant positive correlation between the number of years that Cry3Bb1-expressing hybrids have been cultivated in a grower's field and the survival of the western corn rootworm populations on Cry3Bb1-expressing seedlings in laboratory plant based bioassays. The corrected survival in Iowa ranged from 17 to 21% for populations from Iowa fields without a history of Cry3Bb1-expressing hybrids (control fields), and between 32 and 62% in performance failure fields where Cry3Bb1 hybrids had been planted for at least 3 consecutive years (Gassmann et al. 2011). This corresponds to the three generations of Cry3Bb1 selection pressure on the laboratory colony described by Meihls et al. (2008). The Illinois Cry3Bb1 performance failure fields, described by Gray (2011b,c), had also been planted with Cry3Bb1 hybrids for three or more consecutive years.

Western corn rootworm resistance to the Cry3Bb1 protein has been described as being inherited non-recessively which means the high dose requirement, foundational for Bt refuge strategies, was not met (Bates et al. 2005; Petzold-Maxwell et al. 2012; Meihls et al. 2008). Meihls et al. (2008) described a mortality estimate of western corn rootworm resulting from Cry3Bb1 consumption to be 96.21%, and others also reported a low mortality to Cry3Bb1 hybrids (Siegfried et al. 2005; Nowatzki et al. 2006; Meissle et al. 2011). Since resistance is not recessive, the concentration of the Cry3Bb1 in Bt

corn root tissue is likely not high enough to kill sufficient numbers of heterozygote offspring (Gould 1998).

Western corn rootworm resistance to the binary Cry34/35Ab1 protein has been observed after eleven generations of selection pressure in a laboratory setting (Lefko et al. 2008). Field estimates of western corn rootworm survival after exposure to Cry34/35Ab1 are 0.6-4.0% (Storer et al. 2006). Hibbard et al. (2010a) determined the corrected (unadjusted) mortality of Storer et al. (2006) to be 96.48%. Field-evolved resistance to mCry3A also has been described for the western corn rootworm (Gassmann et al. 2014). After four and seven generations of selection, survival of resistant western corn rootworms was similar on mCry3A hybrids and the near-isogenic line in the greenhouse and field, respectively (Meihls et al. 2011). Hibbard et al. (2010b, 2011) estimated western corn rootworm mortality to mCry3A in a field setting to be 94.88% and 97.83%, respectively. The US EPA (2007) reported that mortality of western corn rootworms to mCry3A hybrids ranged between 89.9% using an artificial western corn rootworm infestation and 92.2% under natural infestations.

The US EPA sets the standard for a high dose Bt-crop at 99.99% mortality (Roush 1994) to ensure high mortality the heterozygotes. The findings previously cited indicate that Cry3Bb1, Cry34/35Ab1, and mCry3A proteins (when expressed as a single toxin) fail to meet the high dose standards used to determine adequate refuges to prevent field-evolved resistance. Instead, data indicate that Bt expression of these commercialized traits are low to moderate in dose, raising doubts as to the future effectiveness of rootworm-Bt technology under current refuge requirements (Tabshnik and Gould 2012). Further compounding these concerns is the higher presence of resistance allele frequency than initially predicted. Onstad and Meinke (2010) predicted that the initial resistance allele frequency in field settings may be as high as 0.2 for the Cry3Bb1 protein and ranges from 0.05 to 0.1 for the Cry34/35Ab1 toxin.

Also challenging the effectiveness of a structured refuge for resistance management is the low level of random mating occurring between resistant and susceptible western corn rootworm adults. In order for the refuge strategy to maximize resistance prevention, susceptible insects emerging from the refuge should outnumber resistant insects, and the emerged resistant/susceptible insects should mate randomly. The range of adult western corn rootworm movement has been shown to be less than 30 m per day (Coats et al. 1987; Nowatzki et al. 2003; Spencer et al. 2003, 2005). Spencer et al. (2009) determined that the average adult dispersal rate is approximately 15 m per day. This typically short movement of adults raises concerns of non-random (assortative) mating among susceptible adults from refuges and potentially resistant adults emerging from Bt hybrids. These concerns call into question the continued value of the structured refuge as a resistance management tactic for the western corn rootworm. Additionally, adults in Bt corn emerge later than those in the refuge also leading to non-random mating (Clark et al. 2012). Murphy et al. (2010) observed a 2 week delay in emergence of western corn rootworm adults from Cry3Bb1 hybrids compared with the near-isogenic line.

Short and long distance dispersal of corn rootworm adults also supports the observation of Cry3Bb1 problem fields arising independently rather than spreading from a central occurrence, thus delaying resistance at the landscape level (Caprio and Tabashnik 1992). However, Gassmann et al. (2011) discussed the theory that independent occurrences of Cry3Bb1 resistance may contribute to the persistence and intensity of resistance in localized areas.

In most published literature that analyzes fitness costs associated with western corn rootworm Bt resistance, the presented data to date suggest little to no fitness costs associated with Cry3Bb1 resistance (Meihls et al. 2008). However, Meihls et al. 2012 did find fitness costs associated with fecundity and the adult male lifespan. Similarly, Gassmann et al. (2012) reported some of the western corn rootworm populations they tested displayed significantly lower survival on non-Bt hybrids than the

control populations. The fact that the majority of data support a lack of fitness costs associated with Cry3Bb1 resistance, indicate that resistance may spread rapidly through a population.

Currently, there are 23 counties in the United States Corn Belt (NE, IA, SD, MN, WI, and IL) with confirmed reports of greater than expected corn rootworm damage (at least one node of roots destroyed) (US EPA 2012). Corn hybrids expressing the Cry3Bb1 (2003) protein were the first commercialized rootworm-Bt products (2003). These corn hybrids were also the first to have product failures develop in a field setting. Corn hybrids expressing the Cry34/35Ab1 (2006) and mCry3A (2007) proteins have since been commercialized and have grown in market share. Since resistance to the Cry34/35Ab1 (Lefko et al. 2008) and mCry3A proteins (Meihls et al. 2011) have both been demonstrated in a laboratory and/or greenhouse setting, the potential for field-evolved resistance is a continuing concern for both industry and growers, and the impressive benefits Bt technologies offer including reduced reliance on conventional insecticides and, in some cases, suppression of western corn rootworms, may soon be limited.

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CHAPTER 2: INVESTIGATIONS OF POTENTIAL FIELD-EVOLVED RESISTANT POPULATIONS OF THE WESTERN CORN ROOTWORM

INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is an economically important pest of corn, *Zea mays* L. in both the United States and Europe (Gray et al. 2009). Control measures combined with yield loss associated with this pest have been estimated to exceed $1 billion annually in the United States (Metcalf 1986).

The western corn rootworm is a univoltine species that consumes primarily corn tissue throughout its life cycle (Chiang 1973). Rootworm larvae that hatch in the spring will immediately feed on corn root tissues; however, roots of other grass species can also support larval growth (Branson and Ortman 1967; Oyediran et al. 2004; Oyediran et al. 2008). Larvae feed on corn roots throughout all three instars of development (Levine and Oloumi-Sadeghi 1991). Their feeding causes damage by disrupting water and nutrient flow (Kahler et al. 1985). Feeding wounds on the roots also create entry points for diseases which further limits the yield potential of a crop (Bryson et al. 1953). Additionally, larvae can cause corn plants to become lodged or "goose-necked" which can also reduce yield by impeding mechanical harvest (Riedell 1990). Larvae pupate in the soil and typically emerge as adults in late June and early July. Adults can generally be found in fields until the first killing frost (Levine and Oloumi-Sadeghi 1991). Peak male emergence occurs approximately 5 days prior to peak female emergence; however, emergence for both sexes overlaps for the majority of the growing season (Branson 1987; Murphy et al. 2010; Quiring and Timmins 1990). Adults feed on corn foliage, silks, tassels, and young kernels.

The western corn rootworm has been managed traditionally through crop rotation and the use of planting-time soil insecticides. In 2003, the United States Environmental Protection Agency approved the first commercial transgenic rootworm Bt (*Bacillus thuringiensis* Berliner) corn hybrids to manage the larvae of this significant pest of corn. Bt hybrids designed to offer corn rootworm protection express

various toxic Cry proteins (i.e., Cry3Bb1, Cry34Ab1/Cry35Ab1, mCry3A, eCry3.1Ab) which have proven to be valuable tools for protecting corn roots from rootworm larval feeding. However, the western corn rootworm has repeatedly developed resistance to management techniques such as crop rotation (by ovipositing in non-corn crops) (Levine et al. 2002; Gray et al. 2009) and some conventional insecticides (Ball and Weekman 1962; Meinke et al. 1998; Wright et al. 2000; Siegfried et al. 2004). The widespread and repeated use of Bt hybrids has allowed for significant selection pressure and has increased the risk of western corn rootworm resistance to Bt (Siegfried et al. 1998).

There have been several cases of field-evolved resistance to Bt crops since initial commercialization, but they have historically only included lepidopteran pests (Tabashnik et al. 2004; van Rensburg 2007; Storer et al. 2010; Downes et al. 2010; Bagla 2010; Dhurua and Gujar 2011). A greenhouse-selection experiment in 2008 found higher survival on Cry3Bb1 corn by western corn rootworm after only three generations of selection pressure, which showed the potential capacity for rapid development of resistance (Meihls et al. 2008). Studies of problem fields in which farmers planted Bt hybrids expressing the Cry3Bb1 protein resulted in the first cases of field-evolved resistance by the western corn rootworm. Within these Iowa fields, Bt hybrids had been used in continuous corn production fields. These cases of field-evolved resistance by the western corn rootworm represent the first coleopteran to develop resistance to a Bt toxin (Gassmann et al. 2011).

The mechanism of resistance within the rootworm is currently unknown; however, there are at least five points in the Bt toxicity pathway where genetic change in an insect could decrease effects of the Bt protein: (1) decreased solubilization of the Bt crystal, (2) decreased cleavage of the full-length Bt protein into an active fragment, (3) increased proteolytic digestion of the active fragment, (4) decreased binding of the active fragment to the midgut epithelium, and (5) decreased functional pore formation (Bauer 1995; Gill 1992).

Current field resistance occurrences have been attributed to a number of factors including the repeated deployment of the same Bt-corn hybrids which increases the selection pressure on the toxin, low grower compliance with refuge requirements, and use of low dose transgenic events. In such a high-selection environment, western corn rootworm resistance can either (1) simultaneously develop in different locations, (2) spread from a resistance hot spot to neighboring corn fields and states, and/or (3) be a combination of both (US EPA 2011).

Insect resistance management procedures for rootworm Bt crops have historically focused on the high dose/refuge strategy to prolong Bt corn effectiveness. Shortcomings associated with this approach include: (1) early commercialized Bt-toxins were expressed at a low to moderate dose; (2) higher than anticipated initial occurrences of resistance alleles in field populations; (3) resistance traits could have non-recessive inheritance; (4) western corn rootworms emerging from refuges and Bt area of fields may mate in a non-random manner; and (5) fitness costs associated with Bt-resistance may not be present in field environments (Devos et al. 2013).

The primary goal of the field experiments described in the remainder of this chapter was to determine if fields in Illinois in which growers had experienced greater than expected levels of root damage and lodging contained populations of western corn rootworms that had evolved resistance to Cry3Bb1. These fields were planted to corn for many consecutive years and the same Cry Bt trait (Cry3Bb1) had been used. The second goal was to determine if there were differences in node-injury ratings and adult emergence among commercially available Bt hybrids at these sites with potential Cry3Bb1 resistance. The final goal was to determine if there were any fitness costs associated with resistance to a Bt toxin. This research will expand the observations of field-evolved resistance to the Cry3Bb1 protein by the western corn rootworm and contribute to the understanding of how resistance to one Bt protein may affect a field population of western corn rootworms in relation to other commercially available Bt proteins.

MATERIALS AND METHODS

Field Sites

Illinois field experiments were established at two sites in 2012 and at three sites in 2013 to evaluate suspected and confirmed resistance to the Cry3Bb1 toxin by western corn rootworm larvae (Figure 1). In 2012, two trials were located in northwestern Illinois. One trial was established north of Lostant (La Salle County, 41.143, -89.060) and a second trial was established south of Prophetstown (Whiteside County, 41.671, -89.936). In 2013, trials were replicated at Lostant and Prophetstown. A third trial was established south of Urbana in 2013 at the University of Illinois agricultural engineering research farm (Champaign County, 40.111, -88.207). This site was added to improve the chances for adequate corn rootworm larval injury and adult emergence due to later than optimal planting at the LaSalle and Whiteside County sites in the spring of 2013. At both northwestern sites, the grower/cooperator planted continuous corn for 3 or more years preceding our research and used the same rootworm-Bt toxin (Cry3Bb1) during each of those years. Additionally, these fields experienced greater than expected root injury in response to WCR larval feeding in 2011.

In 2013, all trials were established in areas of these growers' fields that in 2012 were planted to corn where cucurbit powder (Sentry Biologicals Inc., Billings, MT) was applied in an attempt to attract ovipositing females. Cucurbitacins are allomones that originally served to protect the plants from attack by herbivores. For a large group of diabroticite beetles, including the western corn rootworm, these allomones have become kairomones which stimulate specific sensory receptors on the maxillary palpi and elicit the beetle response of arrest and compulsive feeding (Ferguson and Metcalf 1985). Approximately four scoops (5.05 kg) were applied on each field by walking through every fifth row of corn and spreading the powder within the furrow at every 20th pace (~15 meters). Growers were not asked questions regarding whether or not they established a refuge in preceding corn on corn years. Bioassay experiments included adult western corn rootworms from the three previously described field

sites (Whiteside, LaSalle, and Champaign Counties), but also included adults from Iowa control fields where injury to Cry3Bb1 corn had not been reported.

Planting

The experimental design was a randomized complete block with four replications. The plot size for each treatment was 3.05 m (four rows) x 9.14 m. In 2012, trials were established on 9 and 16 May at Prophetstown and Lostant, respectively. In 2013, trials were established on 13 May, 30 May, and 5 June at Prophetstown, Urbana, and Lostant, respectively. Trials were planted using a four-row, vacuum style planter constructed by Seed Research Equipment Solutions (SRES, South Hutchinson, KS). Seeds were planted in 76.2-cm rows at an approximate depth of 4.45 cm. Standard agronomic maintenance practices were performed.

An additional area was planted at each site in both 2012 and 2013. This area was used solely for beetle collection for plant-based bioassays. The collection area was 3.05 m (four rows) x 15.24 m.

Treatments

In 2012, and 2013; eight unique hybrids commonly available to growers were used for these experiments. These hybrids included rootworm single Bt toxins, rootworm pyramided toxins, and untreated checks (non-rootworm Bt, near isolines) that corresponded to the single trait and/or pyramided family of Bt toxins. These treatments with the associated rootworm Bt toxin(s) in parenthesis are as follows: (1) Herculex XTRA (Cry34/35Ab1), (2) Herculex I (untreated check), (3) Agrisure RW (mCry3A), (4) Agrisure 3122 (Cry34/35Ab1 and mCry3A), (5) Agrisure GT (untreated check), (6) SmartStax (Cry34/35Ab1 and Cry3Bb1), (7) YieldGard VT3 (Cry3Bb1), and (8) Roundup Ready 2 (untreated check).

Bt treatments did not include refuge seed, and non-experimental seed was factory treated with a neonicotinoid seed treatment, ‘Cruiser’ or ‘Cruiser Extreme’ (thiamethoxam, Syngenta Crop Protection, Wilmington, DE) at a rate of 0.25 mg AI/kernel.

Sampling

To determine if the western corn rootworm population in our field sites was resistant to the Cry3Bb1 protein, we collaborated with Dr. Aaron Gassmann of Iowa State University who maintains an insectary equipped for plant based bioassays used for Bt resistance experiments.

Adults for the bioassays were collected using two 12 ft (3.66 m) x 12 ft (3.66 m) walk-in style screenhouses (Model no. 70711010, Kelty, Boulder, CO)(Figure 2) placed in an area that had been planted to a non-rootworm Bt hybrid (Herculex I, Agrisure GT and Roundup Ready 2). Live beetles were collected from the tents and placed in 16 oz (0.35 L) clear wide mouth plastic jars (Olcott Plastics, St Charles, IL) along with corn rootworm diet (Western Corn Rootworm Diet Without Pollen Substitute, BIO-Serve, Frenchtown, NJ). Jars were then placed in 33.02x33.02x31.75 cm insulated shipping kits (Uline, Pleasant Prairie, WI) and shipped, overnight to Dr. Aaron Gassmann's laboratory (Iowa State University, Ames, IA). Adults were collected 9-12 July for 2012 experiments and 7 July and 8 August for 2013 experiments. At the Iowa State Insectary, procedures were used according to those reported by Gassmann et al. (2011) for single plant bioassays.

Upon arrival at the Iowa State Insectary, adult western corn rootworms were held in small cages where they were provided with food consisting of corn leaf tissue and an artificial diet (western corn rootworm diet, Bio-Serv, Frenchtown, NJ). The water source for the beetles was 1.5% agar solid, which was 98.5% water by mass. Cages were held in an incubator (25^{o}C) and individuals from each population were held in separate cages. Adults were provided with an oviposition substrate that consisted of moist, finely sieved soil placed in a 10 cm Petri dish. Eggs obtained from each population were placed separately in 45 ml plastic cups containing moistened sieved soil, and then placed in cold storage for at least 5 months to break diapause. Following exposure to cold, eggs were stored for 1 week at 25^{o}C. Eggs were washed from the soil and placed upon moistened sieved soil and held in a 10 cm Petri dish. Neonate larvae began hatching approximately 1 week thereafter. Neonate larvae from each population

were evaluated in laboratory bioassays for their survival on two transgenic corn hybrids, each of which expressed a unique Bt toxin targeting corn rootworm. One hybrid (DeKalb DKC 6169) expressed the Cry3Bb1, and the other hybrid (Mycogen 2T789) expressed the Cry34/35Ab1 toxin. For both Bt hybrids, rootworm survival was also evaluated on a near isogenic hybrid (non-Bt hybrid). For each site population submitted to Iowa State University, eight total single plant bioassays (Eight replicates for Bt hybrids and eight replicates for non-Bt untreated checks) were tested.

For greenhouse bioassays, plants (at the V5 stage) were trimmed to a height of 20 cm to allow for storage in incubators. Recently hatched larvae (less than 24 hours old) were placed at the base of a corn plant on a root. Between 10 and 20 neonates were placed at the base of each plant. Cups containing plants and larvae were placed in an incubator for 17 days (25^{o}C, 65% RH). After 17 days, the aboveground plant biomass was excised and the soil containing the roots and larvae was removed and placed on a Berlese funnel to extract larvae from the soil. Root masses were held on Berlese funnels over 4 days, and rootworm larvae were collected in 15 ml glass vials containing 10 ml of 85% ethanol. Survivorship and mortality were then analyzed for each site along with a control population in which the Cry3Bb1 protein was still effective.

Injury to corn roots caused by western corn rootworm larvae among treatments was conducted in 2012 and 2013. Five randomly selected root systems were extracted from the second row of each plot on 16 and 17 July at Lostant and Prophetstown, respectively, in 2012. In 2013, five randomly selected root systems were again extracted from the second row of each plot on 22 July, 5 August, and 6 August at Prophetstown, Lostant, and Urbana, respectively. The root systems were washed and rated at the University of Illinois Agricultural Engineering Farm (Urbana, IL) for corn rootworm larval injury using the 0 (no injury) to 3 node (maximum feeding injury) scale (Oleson et al. 2005). This scale is based on the proportion of nodal roots that display larval feeding injury. A root is considered pruned if it has been chewed back to within 3.8 cm of the corn stalk.

To investigate emergence and fitness measurements, three Illinois-style single-plant emergence cages as described by Pierce and Gray (2007) (Figure 3) were deployed and spaced evenly in the first row of each plot at the Prophetstown and Lostant sites in 2012 and at the Prophetstown and Urbana sites in 2013. These cages were deployed prior to adult western corn rootworm emergence, and spanned row one of each plot over one healthy corn plant (V5-V7) per cage. Because the seed used had no refuge, it was assumed that every selected plant expressed the expected Bt protein, excluding those untreated check treatments which were void of rootworm Bt toxins. The top of a young corn plant was inserted through a hole at the top of the emergence cage. An inverted glass jar was placed into the other hole at the top of the cage. Upon emergence, rootworms naturally move upward due to negative geotaxis. Because of this, beetles congregate inside the glass jar, which is the highest point they can reach within the cage. An inverted paper cup with its point removed was placed within the jar. The beetles moved through the cup to enter the jar and were then trapped within the jar. Beetles were collected twice per week (Monday and Thursday) from 28 May through 23 August in 2012 and from 5 July though 5 September in 2013. Beetles collected from each of the three cages within a plot were combined into a plastic bag (Uline, Pleasant Prairie, WI) which was labeled and placed in -18^{o}C freezer storage for further processing.

The sex of adults collected from emergence cages was determined in a laboratory setting after emergence concluded to determine the percentage of male versus female emergence. Sex was determined by the examination of the abdominal apex (White 1977). Beetles were then counted to observe different emergence patterns among the treatments. Each adult head capsule was measured in millimeters using a microscope (Model MZ125, Leica Microsystems, Buffalo Grove, IL) at 8X magnification. Beetles were then dried for 24 hours at 60^{o}C in an incubator oven (THELCO Model 28, Precision Scientific, Chicago, IL). Beetle dry weights were then measured using an analytical balance (Model XS104, Mettler-Toledo LLC, Columbus, OH) providing an accuracy of 0.1 mg.

The percentage of lodged plants (plants leaning at 45° or less from the soil surface) was determined on 16 and 17 July at Lostant and Prophetstown, respectively. In 2013, the percentage of lodged plants was determined on 18 September, 19 September, and 1 October at Urbana, Lostant, and Prophetstown, respectively.

Statistical Analysis

Single plant bioassay experiments were compared using a Welch's *t*-test, which is designed for unequal sample sizes with unequal variances. The paired-sample *t*-test was conducted to compare western corn rootworm average mortality in plants expressing the Cry34/35Ab1 protein and plants expressing the Cry3Bb1 protein for the Whiteside County and LaSalle County sites. This two sample *t*-statistic is a tool used to determine if western corn rootworm populations are resistant to the Cry3Bb1 protein, and is calculated as:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}}} \text{ where, } S_{X_1X_2} = \sqrt{\frac{1}{2}(s_{X_1}^2 + s_{X_2}^2)}.$$

Additionally, degrees of freedom were calculated using the following formula from Niaman et al. 1996:

$$d.f. = \frac{(s_1^2/n_1 + s_2^2/n_2)^2}{\left(\frac{s_1^2}{n_1}\right)^2/(n_1 - 1) + \left(\frac{s_2^2}{n_2}\right)^2/(n_2 - 1)}.$$

Variables measured included: node-injury rating, emergence, adult head capsule widths and dry weight. Data was analyzed using SAS 9.3 (Copyright© 2002–2011 SAS Institute, Inc., Cary, NC). In order to stabilize variances and meet the requirements of analysis of variance, all variables were analyzed using a square-root transformation. Statistical tests for fixed effects were performed using PROC MIXED. Fixed effects were declared significant at $P \leq 0.05$. The Kenward-Roger adjustment was used to approximate denominator degrees of freedom (df_D) (Kenward and Roger 1997).

RESULTS

Plant Based Bioassays

Plant-based bioassays were completed at Iowa State University from 2012 through 2014 for several Illinois Cry3Bb1 problem field locations. This analysis is focused on the results from Whiteside and LaSalle Counties. Bioassays of the Champaign population were still ongoing at the time of preparation. A Welch's *t*-test was conducted to compare the corrected larval morality of Cry3Bb1 expressing hybrids and Cry34/35Ab1 expressing hybrids at both sites (Welch 1947). In both the LaSalle ($t = 3.672$, 7 d.f., $P=0.0079$) and Whiteside County ($t = 7.187$, 7 d.f., $P=0.0002$) plant based bioassays, the effect of treatment was significant (Table 1). Treatment 1 consisted of plants expressing Cry3Bb1 protein and treatment 2 consisted of plants expressing the Cry34/35Ab1 protein. Western corn rootworm larval mortality on Cry3Bb1-expressing root tissue was significantly lower than larval mortality on Cry34/35Ab1-expressing root tissue (Figure 4). Corrected mortality at the LaSalle County site was 0.33 on Cry3Bb1 expressing hybrids and 1.00 on Cry34/35Ab1 expressing hybrids (Figure 4). Corrected mortality at the Whiteside County site was 0.461 on Cry3Bb1 expressing hybrids and 0.98 on Cry34/35Ab1 expressing hybrids (Figure 4). Corrected mortality was 0.979 for hybrids expressing the Cry3Bb1 protein and 0.980 for hybrids expressing the Cry34/35Ab1 proteins for larvae that came from the control populations (Figure 4).

These results indicate that western corn rootworm populations in both Illinois problem fields (Whiteside County and LaSalle County) are Cry3Bb1 resistant but the Cry34/35Ab1 protein continues to provide effective western corn rootworm control. Experiments for the Champaign County site are still in progress.

Node Injury Ratings

Western corn rootworm pressure was variable by year and site. In 2012, the effect of treatment was significant on node-injury ratings at LaSalle County (F = 1.34; df = 7, 20.9; *P* = 0.02) but was not significant at Whiteside County (F = 3.19; df = 7, 21.0; *P* = 0.28) (Table 2).

The average amount of root feeding injury observed in Cry3Bb1 problem fields was minimal and never exceeded 0.07 at the LaSalle County site in 2012 (Figure 5). The low amount of injury was likely caused by the later than normal planting dates resulting from a wet spring. Mean node injury ratings at the LaSalle County site in 2012 did have statistically significant differences; however, the differences are unlikely to be of much biological significance due to the low overall levels of damage (Figure 5).

In 2013, the effect of treatment was significant at LaSalle County site (F = 14.23; df = 7, 21.8; *P* = <0.0001) and Whiteside County site (F = 5.68; df = 7, 21.0; *P* = 0.0009) (Table 2) The effect of treatment was not significant at the Champaign County site (F = 0.54; df = 7, 21.0; *P* = 0.79) (Table 2). Experiments in 2013 show much higher mean node injury ratings at the LaSalle County and Whiteside County sites when compared with 2012 data(Figures 6 and 7). However, little root damage was observed at the Champaign County site which may be attributed to a combination of waterlogged soils at larval hatch and later than optimal planting.

In 2013, both the LaSalle County and Whiteside County sites experienced significant differences in node-injury ratings (Figures 6 and 7). At the LaSalle County site, hybrids expressing the Cry34/35Ab1 protein had significantly less damage than the corresponding untreated checks. The hybrid expressing the single mCry3A toxin had statistically similar root injury to its untreated check (B) (Figure 6). At the Whiteside County site, a similar trend was observed with hybrids expressing Cry34/35Ab1 having significantly lower node-injury ratings than the corresponding untreated checks (Figure 7). Additionally, hybrids expressing the single mCry3A toxin and the single Cry3Bb1 protein had statistically similar node-injury ratings to the corresponding untreated checks (B and C, respectively); however, the Cry3Bb1

single toxin treatment also had statistically similar node-injury to the Cry3Bb1 + Cry34/35Ab1 pyramid treatment (Figure 7).

Cumulative Emergence

In 2012, the effect of treatment was significant on male emergence at the LaSalle County site (F = 2.39; df = 7, 21.0; *P* = 0.05) and the Whiteside County site (F = 6.00; df = 7, 21.0; *P* = 0.0006) (Table 2). Means for total seasonal averages of male emergence at the 2012 Whiteside County and LaSalle County sites are reported in Figures 8 and 9. In LaSalle County the hybrids expressing the mCry3A protein has significantly greater mean cumulative male emergence than the corresponding untreated check (B) and the hybrids expressing the Cry34/35Ab1 + mCry3A pyramid (Figure 8). At the Whiteside County site, the mean numbers of males recovered per plot for treatments that had hybrids expressing the Cry34/35Ab1 protein were statistically lower than treatments that had hybrids which did not express the Cry34/35Ab1 protein (Figure 9). The hybrids that expressed the mCry3A protein and its untreated check (B) had statistically similar male emergence (Figure 9). The hybrids that expressed the Cry3Bb1 protein and its untreated check (C) also had statistically similar male emergence (Figure 9).

In 2013, the effect of treatment was significant on male emergence at the Champaign County site (F = 3.01; df = 7, 21.0; *P* = 0.02) and the Whiteside County site (F = 4.87; df = 7, 21.0; *P* = <0.01) (Table 2). At the Champaign County site, male emergence was low and highly variable (Figure 10), but there were significant differences in beetle collections from hybrids expressing both Cry3Bb1 as a single toxin and when pyramided with Cry34/35Ab1 compared with the untreated check (C) (Figure 10). However beetle emergence for the various treatments was low. The same factors that resulted in minimal root injury at the Champaign site likely also contributed to low emergence. At the Whiteside County site, the mean numbers of males collected per plot for hybrids which expressed the Cry34/35Ab1 toxin were statistically lower than the corresponding untreated checks (A, B, and C) (Figure

11). Male emergence at the Whiteside County site in 2013 was not different between hybrids expressing only Cry3Bb1 and mCry3A proteins compared with their untreated checks (B and C) (Figure 11).

In 2012, the effect of treatment was not significant on female emergence for the LaSalle County site (F = 2.01; df = 7, 21.0; *P* = 0.10) or the Whiteside County site (F = 1.88; df = 7, 21.0; *P* = 0.12) (Table 2). In 2013, the effect of treatment was not significant on female emergence for the Champaign County site (F = 1.46; df = 7, 21.0; *P* = 0.24) but was significant for the Whiteside County site (F = 3.95; df = 7, 21.0; *P* = <0.01) (Table 2). At the Whiteside County site, significantly fewer females were collected from plots containing pyramided hybrids (Cry34/35Ab1 with mCry3A or Cry3Bb1) than plots containing the corresponding single toxin hybrids (mCry3A or Cry3Bb1) (Figure 12). Female emergence at the Whiteside County site in 2013 was not different between hybrids expressing only Cry3Bb1 and mCry3A proteins compared with their untreated checks (B and C) (Figure 12).

Male Head Capsule Width

In 2012, the effect of treatment was not significant on mean male head capsule width at the LaSalle County site (F = 0.95; df = 7, 21.0; *P* = 0.49), but the Whiteside County site did experience a significant effect of treatment on mean male head capsule width (F = 12.16; df = 7, 19.1; *P* = <0.01) (Table 2). Mean head capsule width results from Whiteside County are reported in Figure 13. At the Whiteside County site, there were significant male head capsule width differences among the treatments. Mean beetle head capsule width from hybrids expressing the Cry34/35Ab1 toxin had significantly smaller head capsules than beetles from hybrids that did not express Cry34/35Ab1 (Figure 13). Males from hybrids expressing the single trait Cry3Bb1 had mean head capsule widths that were statistically similar to those beetles from their untreated check (C) (Figure 13).

In 2013, the effect of treatment was not significant on mean male head capsule width at the Champaign County site (F = 1.00; df = 7, 10.0; *P* = 0.49), or at the Whiteside County site (F = 0.19; df = 7, 20.0; *P* = 0.98) (Table 2).

Female Head Capsule Width

In 2012, the effect of treatment was not significant on mean female head capsule width at the LaSalle County site (F = 1.69; df = 7, 21.0; *P* = 0.17), but the Whiteside County site did experience a significant effect of treatment on mean female head capsule width (F = 3.19; df = 7, 21.0; *P* = 0.02) (Table 2). At the Whiteside County site there were differences among the treatments (Figure 14). Adults emerging from hybrids expressing the Cry34/35Ab1 toxin pyramided with either mCry3A or Cry3Bb1 had significantly smaller head capsules than treatments that had hybrids that did not express the Cry34/35Ab1 protein in a pyramid (Figure 14). Mean head capsule width of beetles emerging from hybrids expressing single toxin Cry34/35Ab1, mCry3A or Cry3Bb1 had statistically similar mean head capsule widths to their untreated checks (A, B, and C) (Figure 14).

In 2013, the effect of treatment was not significant on mean male head capsule width at the Champaign County site (F = 0.79; df = 7, 16.0; *P* = 0.61), or at the Whiteside County site (F = 0.95; df = 7, 20.0; *P* = 0.49) (Table 2).

Male Beetle Dry Weight

In 2012, the effect of treatment was not significant on mean male weights at the LaSalle County site (F = 0.53; df = 7, 21.0; *P* = 0.81), or at the Whiteside County site (F = 1.45; df = 7, 19.3; *P* = 0.24) (Table 2). In 2013, the effect of treatment was not significant on mean male weights at the Champaign County site (F = 1.36; df = 6, 9.0; *P* = 0.32), or at the Whiteside County site (F = 1.51; df = 7, 20.0; *P* = 0.22) (Table 2).

Female Beetle Dry Weight

In 2012, the effect of treatment was significant on mean female weights at the LaSalle County site (F = 2.62; df = 7, 21.0; *P* = 0.04), but the Whiteside County site did not experience a significant effect of treatment on mean female weights (F = 0.52; df = 7, 21.0; *P* = 0.81) (Table 2). Mean female beetle dry

weights from 2012 at the LaSalle County site are reported in Figure 15. At the LaSalle County site, female beetle dry weight results were highly variable and no clear trend was discernible (Figure 15).

In 2013, the effect of treatment was not significant on mean female weights at the Champaign County site ($F = 0.25$; df = 7, 16.0; $P = 0.07$), or at the Whiteside County site ($F = 2.16$; df = 7, 21.0; $P = 0.08$) (Table 2).

DISCUSSION

With an extensive history of overcoming management practices (Ball and Weekman 1962; Gassmann et al. 2011; Gray et al. 2009; Meinke et al. 2009), western corn rootworm resistance management with regard to Bt hybrids must be a top priority for industry, growers, and regulators. Survival of western corn rootworm larvae on Cry3Bb1 root tissue in single plant bioassays was significantly higher for insects from Illinois problem fields where growers reported greater than expected root injury in 2011 to a Cry3Bb1 hybrid than from control fields not associated with unexpected feeding by western corn rootworms on Bt corn (Figure 4). These results indicate that the western corn rootworm has evolved resistance to the Cry3Bb1 protein in some Illinois populations. These results are similar to the first described case of field-evolved resistance to Cry3Bb1 in Iowa as described in Gassmann et al. (2011). Our data did not detect cross resistance between the Cry3Bb1 and Cry34/35Ab1 proteins.

Once results from ongoing plant-based bioassays are available, we may be able to identify a correlation between the number of years that Cry3Bb1 expressing corn is grown and the survival of western corn rootworms on Cry3Bb1 expressing corn. Gassmann et al. (2011, 2012) found that there was a significant correlation between the number of years that Cry3Bb1 hybrids were grown and the survival of western corn rootworm in the single-plant bioassays.

Node-injury rating scores were very low in 2012, but larval pressure was high in 2013 at the LaSalle and Whiteside County sites. A benefit of low node-injury results may provide would be the lack of density dependent issues that may affect adult emergence results (Hibbard et al. 2004). However, male and female adult emergence trends were similar in 2012 and 2013 at the Whiteside County site which may reflect low density dependent effects.

Some western corn rootworm survival on Bt hybrids is always expected, particularly if the hybrids express a single rootworm Bt protein. Hybrids expressing a single rootworm Bt protein are not considered high-dose (Gassmann et al. 2011; 2012). Since we tested hybrids expressing Cry3Bb1, Cry34/35Ab1, and mCry3A, we can contrast beetle emergence, fitness costs, and node injury ratings among different corn rootworm Bt hybrids. Gassmann et al. (2014) recently documented Cry3Bb1 and mCry3A cross resistance in the field. Node injury scores from this study suggest that Cry3Bb1 and mCry3A cross resistance may also be present in some Illinois field populations. Since Cry34/35Ab1 has a somewhat unique mode of action compared with both Cry3Bb1 and mCry3A proteins, cross resistance with Cry34/35Ab1 is unlikely at sites where field-evolved resistance occurs to Cry3Bb1. Gassmann et al. (2011) found that western corn rootworms resistant to Cry3Bb1 did not exhibit cross resistance to Cry34/35Ab1, nor did this study detect evidence of that cross resistance.

The male emergence levels of western corn rootworms were significantly affected by the treatments in 2012 and 2013 (Table 2). In 2012 at the LaSalle County site, the Cry3Bb1 family of treatments (Cry34/35Ab1 + Cry3Bb1, Cry3Bb1, untreated check C) showed stair stepping among the treatments with the hybrids expressing the Cry3Bb1 protein having statistically similar male emergence to both the hybrids expressing Cry34/35Ab1 + Cry3Bb1 and the untreated check (C) (Figure 8). A western corn rootworm population resistant to the Cry3Bb1 protein may produce similar numbers of beetles compared with a non-Bt isoline. This could be a result of incomplete resistance. Emergence patterns do not definitively describe resistance; rather they are another metric that can be evaluated to more

completely characterize a resistant population. We did observe similar beetle emergence patterns between mCry3A and Cry3Bb1 treatments. This may indicate trouble for Illinois farmers who have populations of western corn rootworms resistant to Cry3Bb1 as Gassmann et al. (2014) describes cross resistance between the mCry3A and Cry3Bb1 proteins.

Adult beetle emergence was lowest for treatments that expressed the Cry34/35Ab1 protein as a single trait or within a pyramid. These results show that in fields with Cry3Bb1 field-evolved resistant western corn rootworms; the Cry34/35Ab1 protein is still effective at reducing beetle survival. However, we did observe similar adult emergence between the Cry34/35Ab1 treatment and its corresponding untreated check, particularly at the LaSalle County site in 2012. These results show that a large number of beetles can emerge from hybrids that effectively prevent root damage. Additionally, Gray et al. (2007) described the potential for significant rootworm damage to Bt hybrids (Cry3Bb1) under very high rootworm pressure, especially later in the summer. The amount of extractable Cry3Bb1 protein declines later in the growing season (Vaughn et al. 2005). Declining toxin levels late in the season could factor into late season injury to hybrids expressing the Cry3Bb1 protein. There is concern about the use of pyramided hybrids that includes Cry3Bb1 as one of the proteins, that the single Cry34/35Ab1 toxin is serving as the primary source of beetle mortality and as such that protein is facing increased selection pressure especially at reduced refuge size in the newly registered Bt hybrids that rely upon seed blends for refuges.

Overarching trends in fitness measurements were difficult to identify for 2012 and 2013 results. The greatest variability in adult head-capsule width measurements occurred among male beetles emerging from the Whiteside county site in 2012 (Figure 13). Within this experiment, hybrids expressing the Cry34/35Ab1 protein, as a pyramid or single toxin, had significantly smaller head capsule widths than their corresponding untreated check which lacked any Bt protein (Figure 13). A similar trend was observed with female beetles in 2012 at the Whiteside County site. Although these results may suggest

that a fitness cost in the form of reduced head-capsule width is associated with beetle emergence on western corn rootworm susceptible Bt hybrids, this trend was not observed at the LaSalle County site in 2012 or at either site in 2013. Few differences in adult beetle dry weight were observed with the exception of LaSalle County female beetles in 2012 (Figure 15). Without a similar trend in fields that have confirmed field-evolved resistance to the Cry3Bb1 protein, we cannot definitively conclude that there are fitness costs associated with Cry3Bb1 resistance, which may have contributed to the speed of evolution of western corn rootworm resistance to the Cry3Bb1 protein in a field setting.

Other factors that may have contributed to field-evolved resistance to the Cry3Bb1 protein include the non-high dose expression of Cry toxins in rootworm-targeting Bt hybrids (US EPA 2010), and evidence that resistance to Cry3Bb1 hybrids in western corn rootworm is not recessive (Meihls et al. 2008). Because the initial commercialized rootworm Bt hybrids expressed a single Cry toxin that was not high dose, original refuge standards were most likely inadequate for the sustainability of the initial rootworm Bt hybrids. Newer rootworm Bt hybrids use pyramided (multiple rootworm Bt products) hybrids which provide a greater level of mortality-therefore, delaying resistance in a field setting if adequate refuge standards are met (Onstad and Meinke 2010). However, Onstad et al. (2011) questions the value of a pyramid strategy for integrated resistance management if multiple-toxin Bt corn fails to increase mortality in the target pest compared with single-toxin Bt corn.

With the widespread use of rootworm Bt hybrids for over a decade, there have been an increasing number of cases of western corn rootworm resistance to Bt. However, these field selected cases of Bt resistance highlights the urgency of developing integrated management solutions for the western corn rootworm. The letter from land-grant University and USDA entomologists to the EPA, Porter et al. (2012) concisely outlines the specific integrated pest management recommendations to help corn producers delay further resistance and conserve western corn rootworm susceptibility to rootworm Bt hybrids:

- Consider rotation to soybean or another non-host crop.
- Consider the use of a corn rootworm soil insecticide at-planting with a non-Bt hybrid.
- Consider the use of a Bt hybrid that expresses a different corn rootworm Cry protein than one which may have performed poorly in the past on a particular farm.
- Consider the use of a pyramided Bt hybrid that expresses multiple Cry proteins targeted against corn rootworms.
- Implement a long tern integrated approach to corn rootworm management that includes previously listed tactics, combined with adult suppression programs where appropriate, and an active field scouting program.

Western corn rootworm management decisions that are made at the farm level have the potential to alter the future progression of rootworm resistance to Bt. Crop scouting and resistance monitoring is important to detect resistance evolution in the field, and integrated pest management is essential to the long-term effectiveness of rootworm Bt corn.

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FIGURES

Figure 1. Location of Illinois field experiment sites (2012 & 2013).

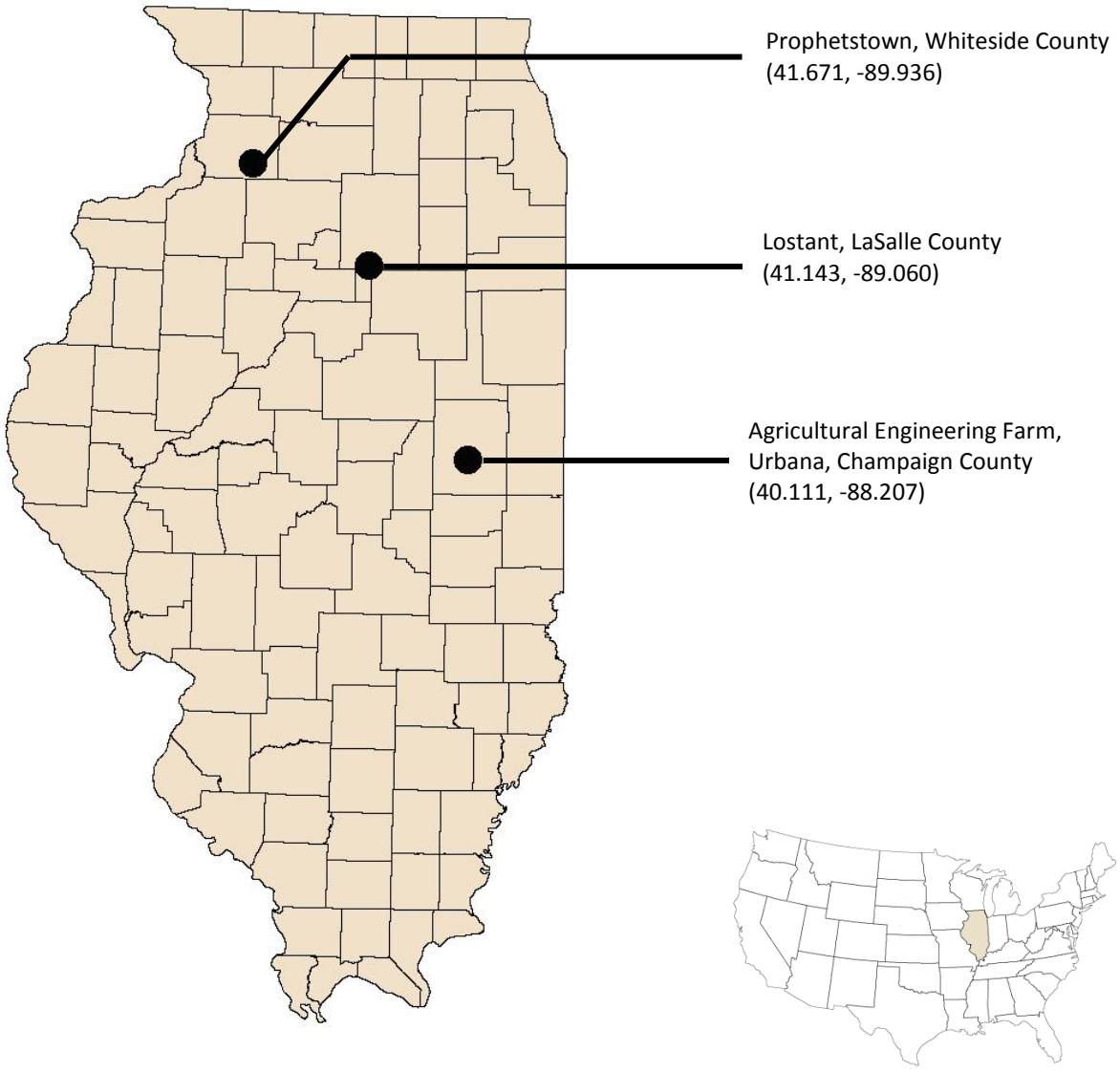
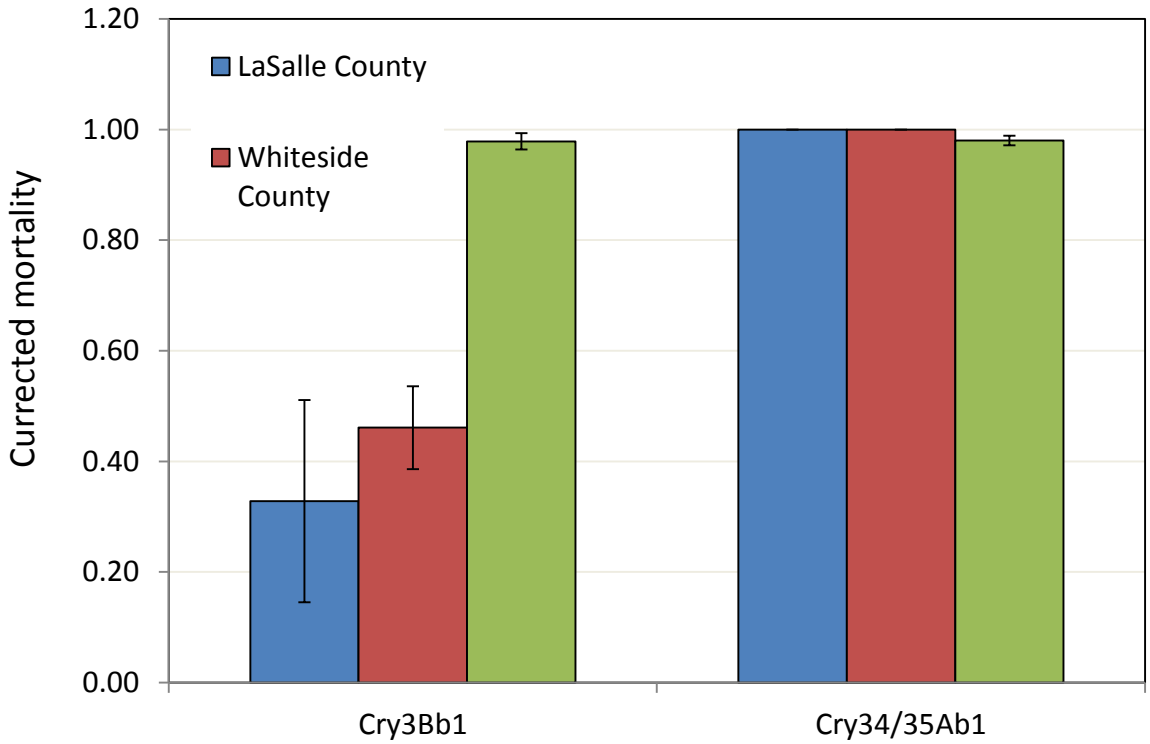


Figure 2. Screenhouses (3.66 m by 3.66 m) used in beetle live capture for plant-based bioassay procedures (2012 & 2013).

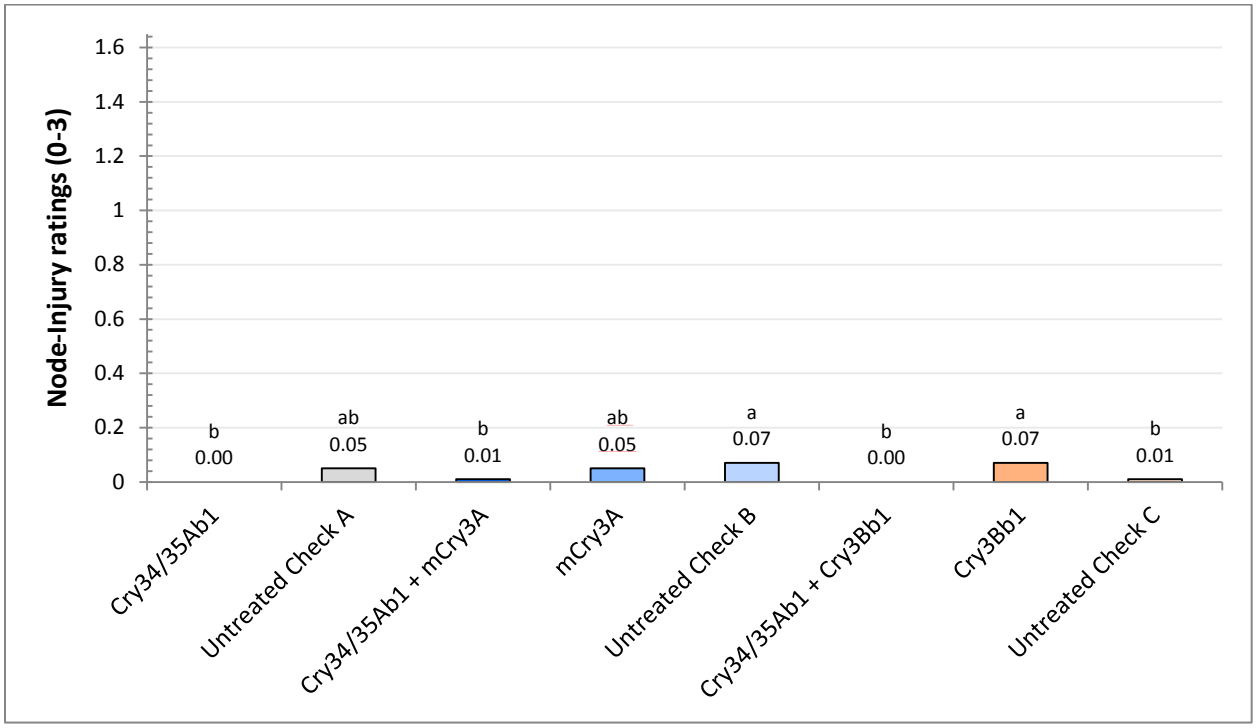
Figure 3. Illinois-style single-plant emergence cage (76.2 cm by 41.5 cm)(2012 & 2013).

Figure 4. Single plant bioassay mortality[1] results for Lostant (LaSalle County) and Prophetstown (Whiteside County), Illinois (2012—2013).



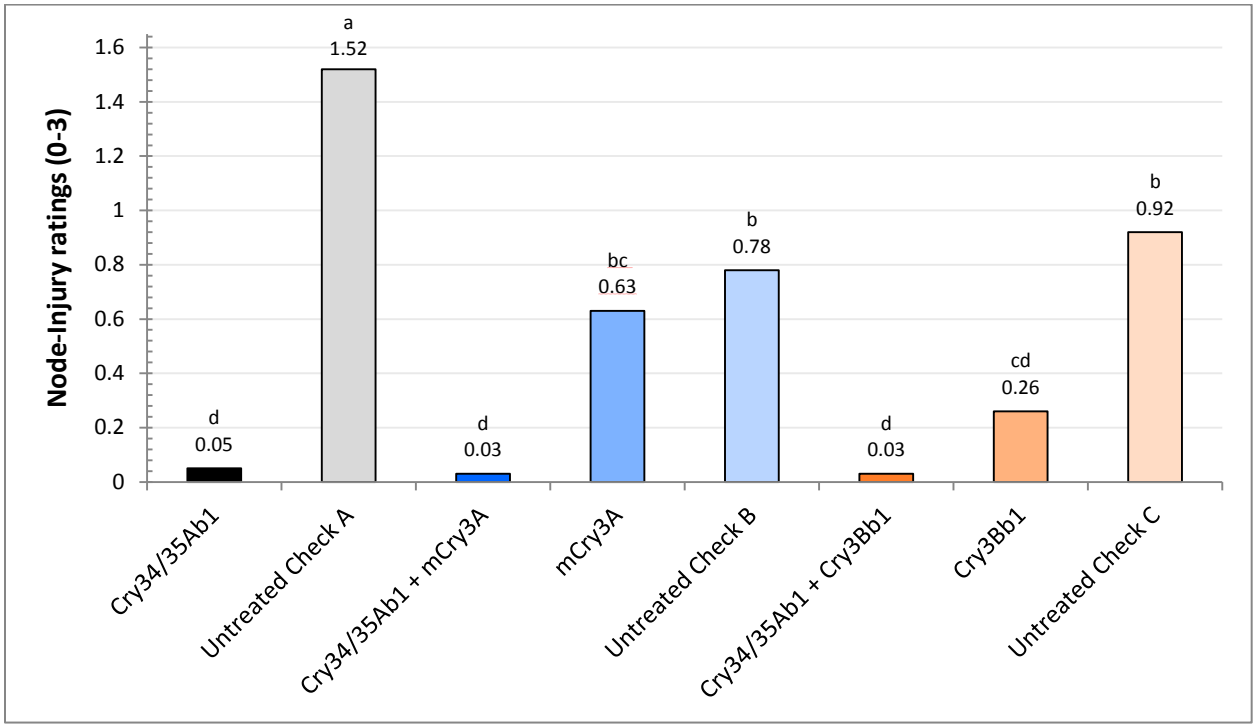
[1]Corrected mortality of western corn rootworm on Bt corn. Mortality data are shown for 1) corn hybrids expressing Cry3Bb1 and 2) corn hybrids expressing Cry34/35Ab1. Bars are sample means and error bars are the standard error of the mean.

Figure 5. Mean[1] node-injury ratings for the effect of treatment, LaSalle County (2012).



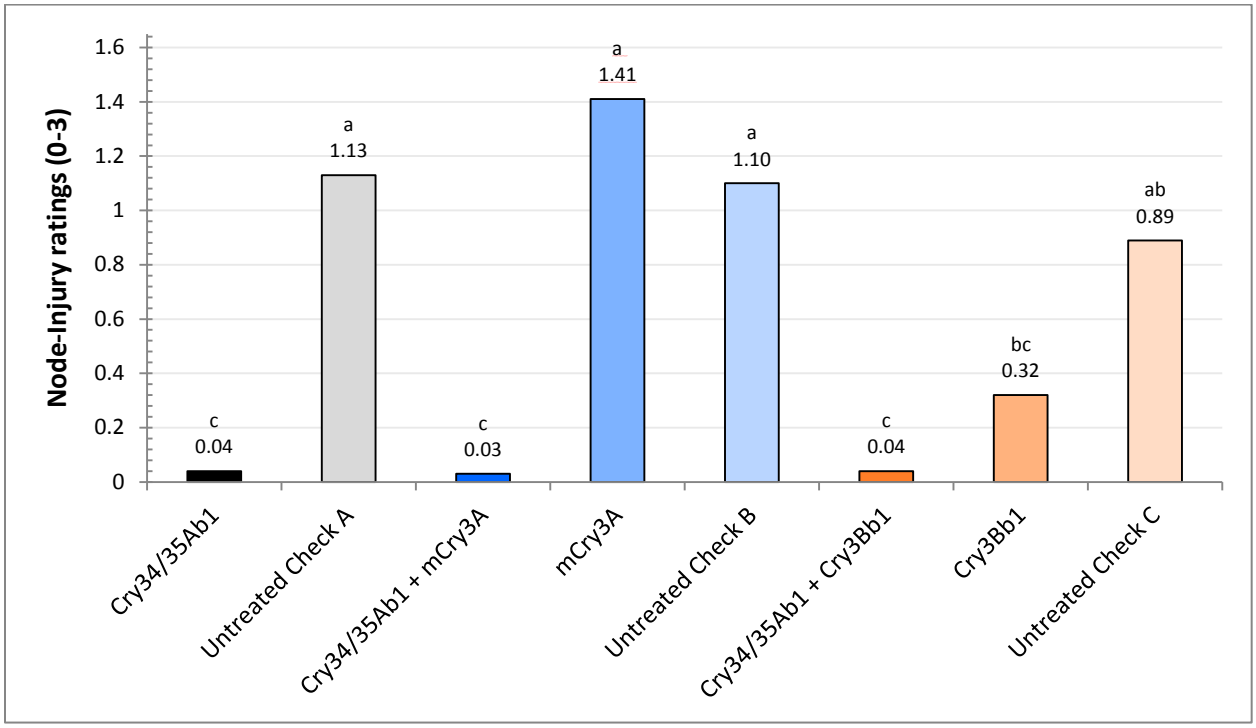
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values were determined using the 0 to 3 node injury scale (Oleson et al. 2005) (NIR = node-injury rating).

Figure 6. Mean[1] node-injury ratings for the effect of treatment, LaSalle County (2013).



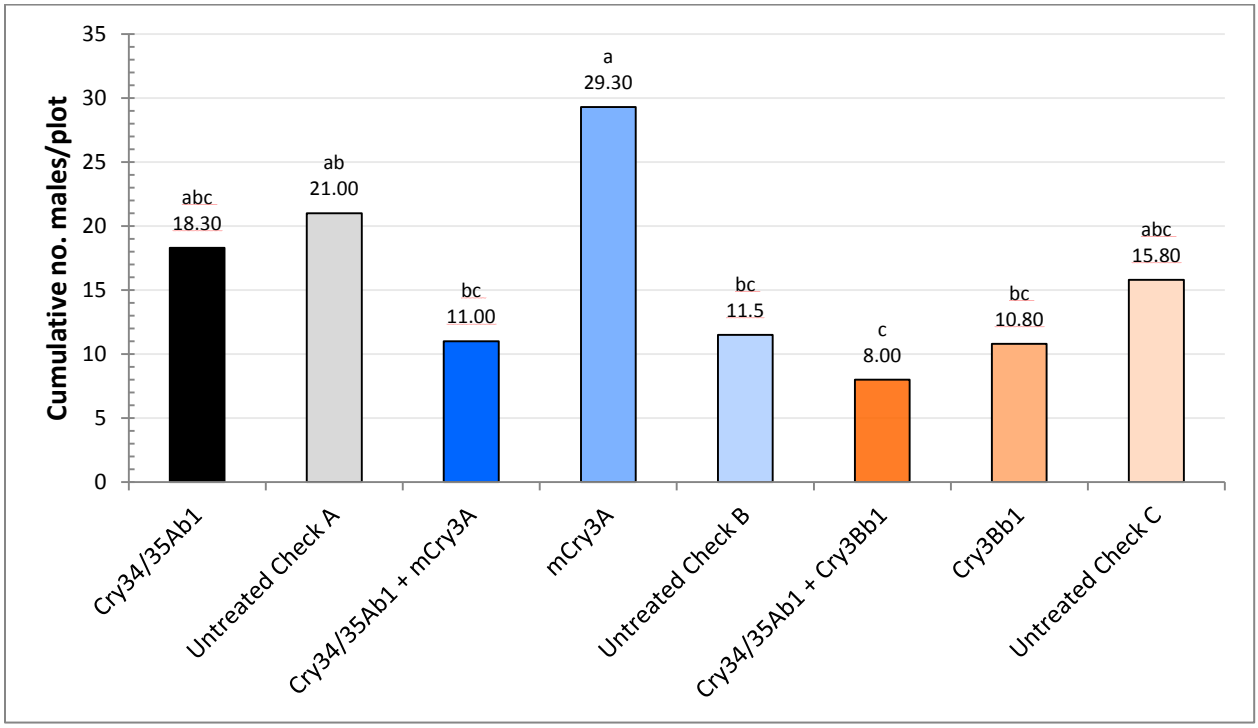
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values were determined using the 0 to 3 node injury scale (Oleson et al. 2005) (NIR = node-injury rating).

Figure 7. Mean[1] node-injury ratings for the effect of treatment, Whiteside County (2013).



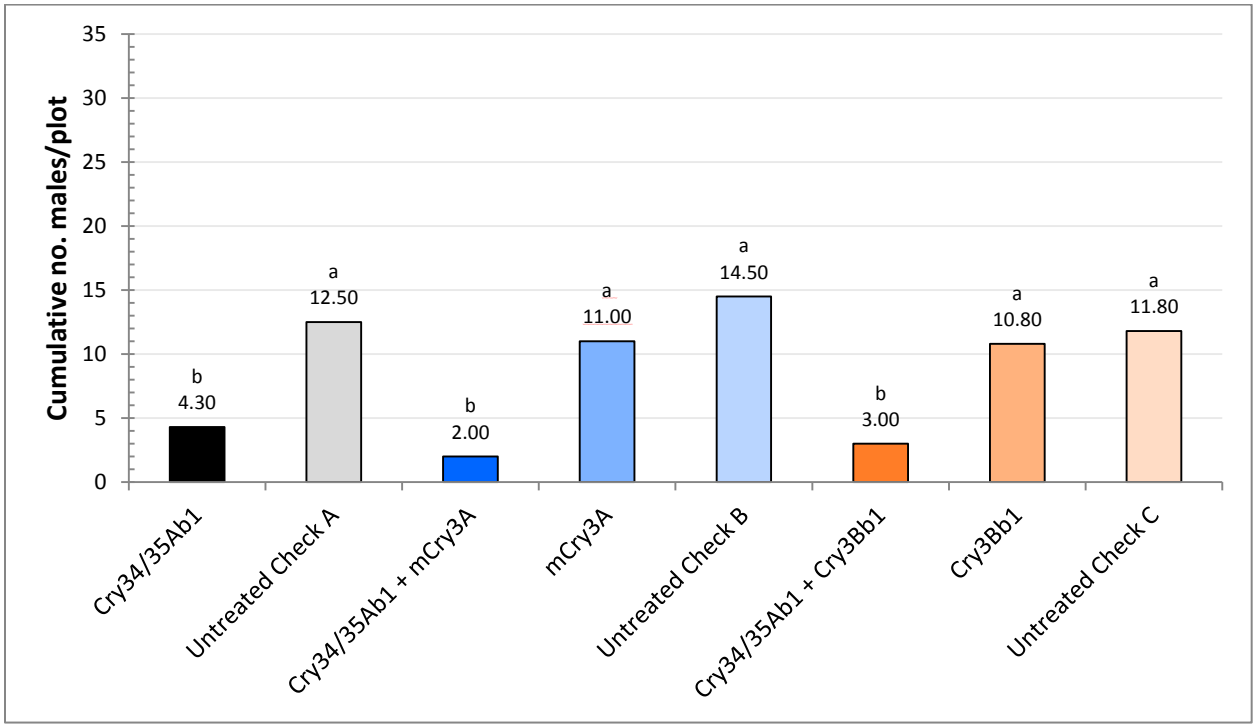
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values were determined using the 0 to 3 node injury scale (Oleson et al. 2005) (NIR = node-injury rating).

Figure 8. Mean[1] male cumulative emergence for the effect of treatment, LaSalle County (2012).



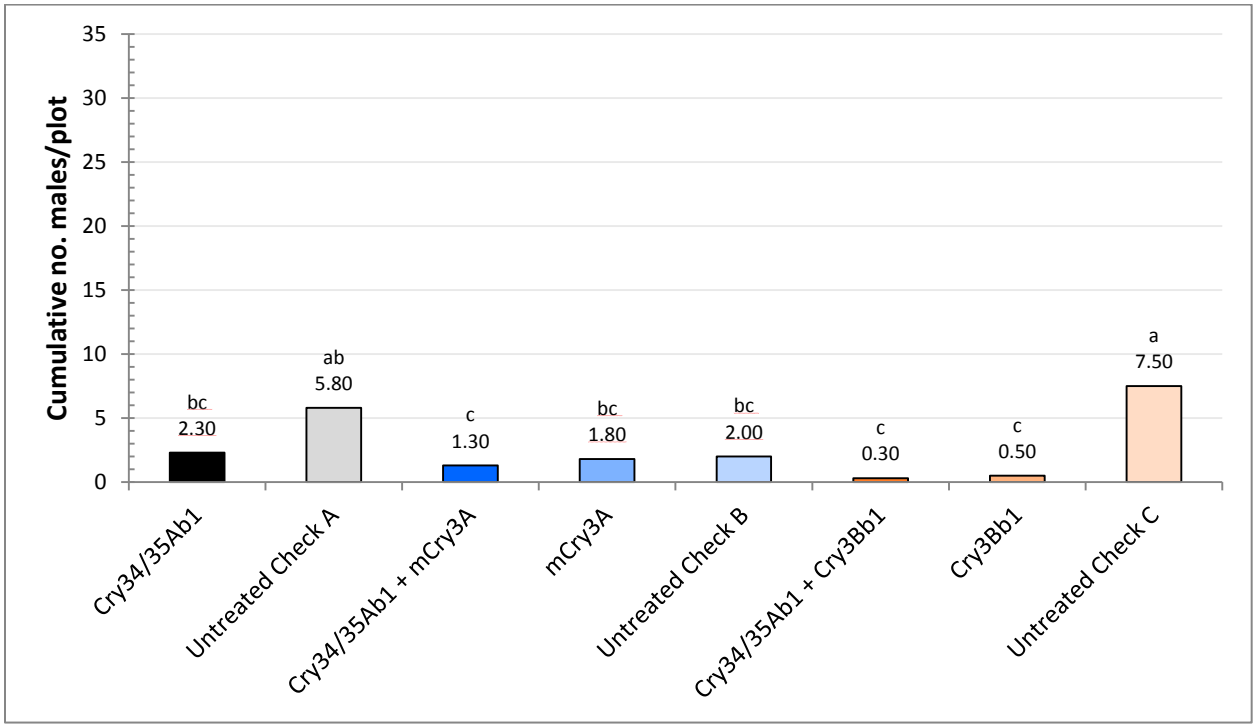
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values represent the cumulative number of beetles captured from three single-plant emergence cages per plot throughout the growing season.

Figure 9. Mean[1] male cumulative emergence for the effect of treatment, Whiteside County (2012).



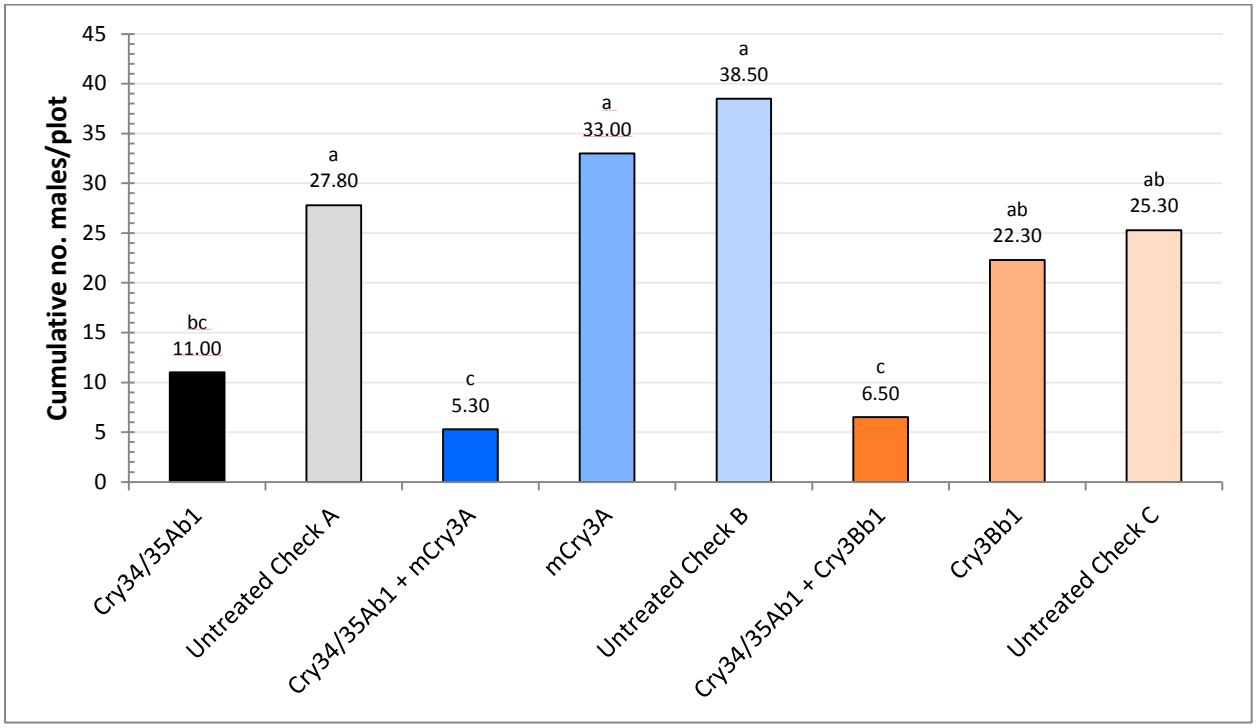
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values represent the cumulative number of beetles captured from three single-plant emergence cages per plot throughout the growing season.

Figure 10. Mean male cumulative emergence for the effect of treatment, Champaign County (2013).



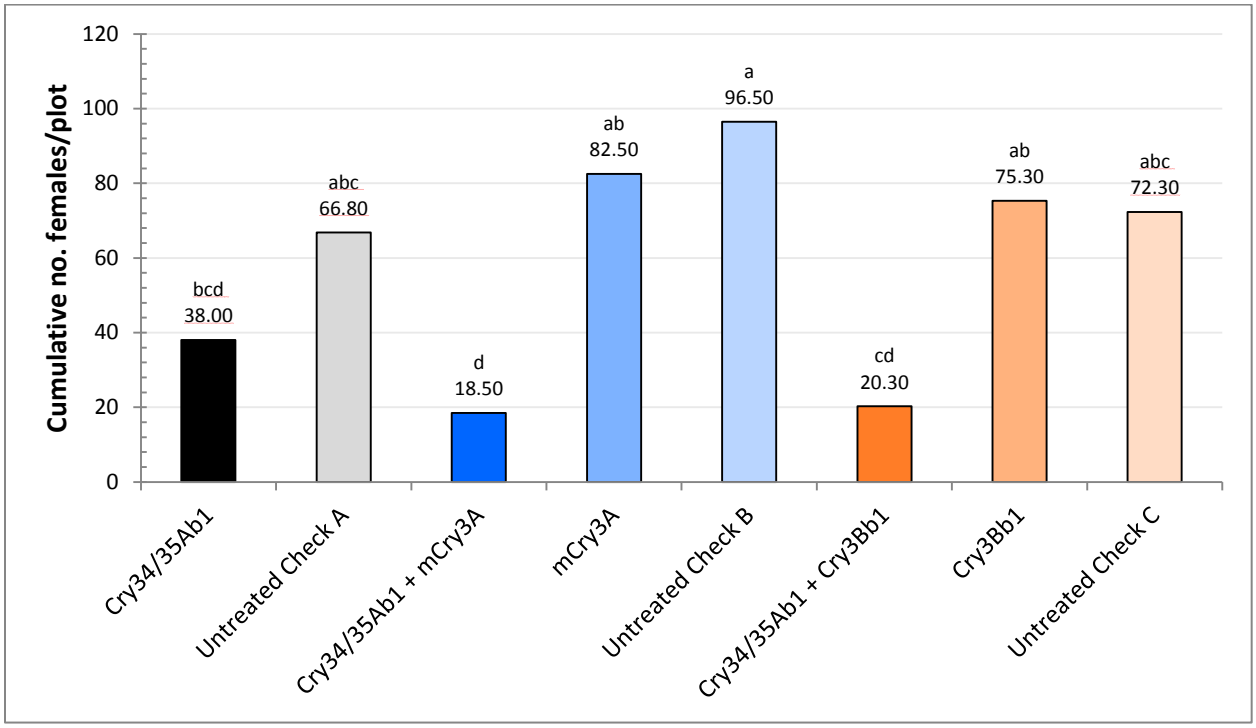
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values represent the cumulative number of beetles captured from three single-plant emergence cages per plot throughout the growing season.

Figure 11. Mean male cumulative emergence for the effect of treatment, Whiteside County (2013).



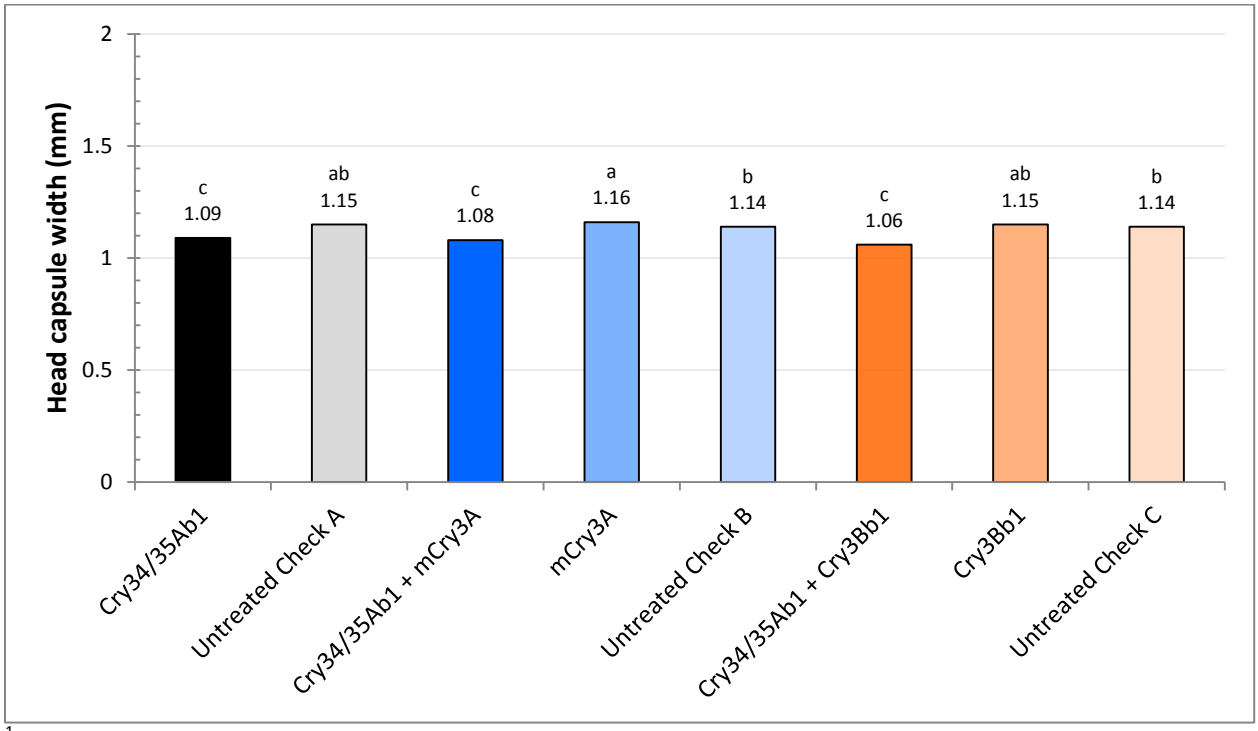
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values represent the cumulative number of beetles captured from three single-plant emergence cages per plot throughout the growing season.

Figure 12. Mean female cumulative emergence for the effect of treatment, Whiteside County (2013).



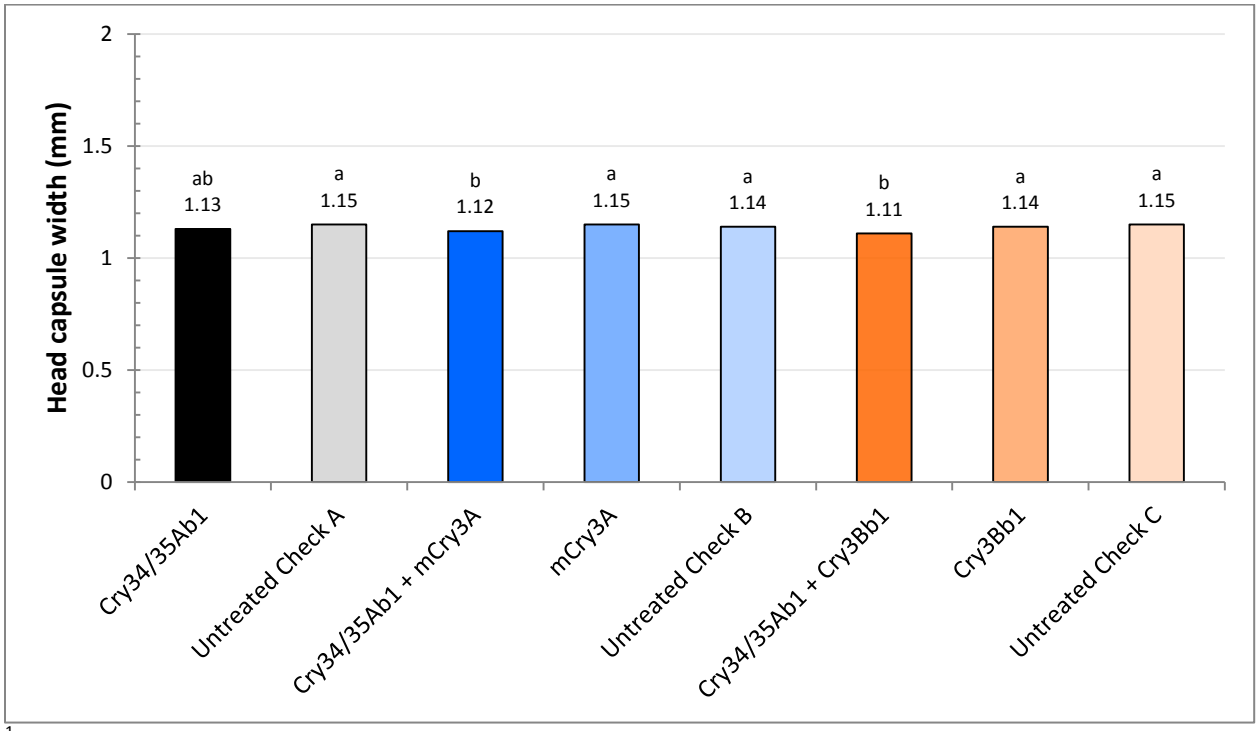
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P≤0.05*). Data were analyzed using a square-root transformation—actual means are reported. [2]Values represent the cumulative number of beetles captured from three single-plant emergence cages per plot throughout the growing season.

Figure 13. Mean[1] male beetle head capsule widths for the effect of treatment, Whiteside County (2012).



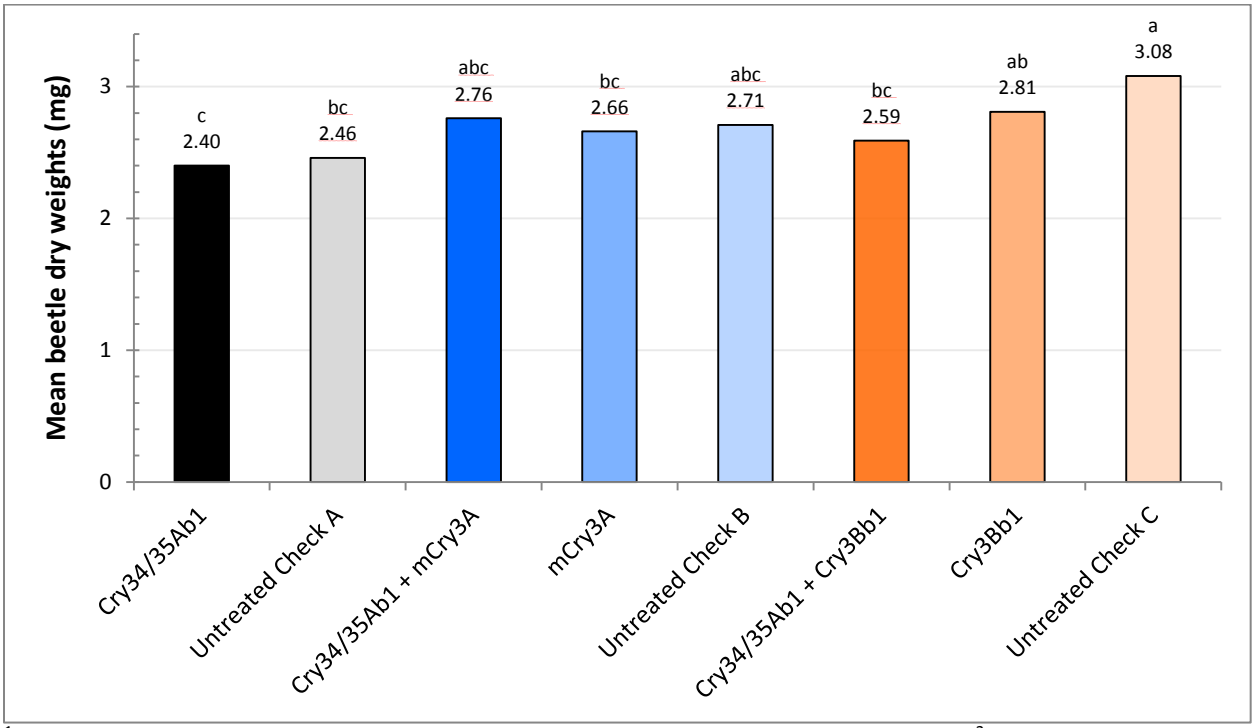
[1]Mean comparisons were performed using PROC MIXED of SAS 9.3. Means with bars that share a similar letter do not differ significantly (*P<0.05*). Data were analyzed using a square-root transformation—actual means are reported.

Figure 14. Mean[1] female beetle head capsule widths for the effect of treatment, Whiteside County (2012).



[1]Means for an individual bar that share a similar letter do not differ significantly (*P<0.05*). Data were analyzed using a square-root transformation—actual means are reported.

Figure 15. Mean[1] female beetle weights[2] for the effect of treatment, LaSalle County (2012).



[1]Means for an individual bar that share a similar letter do not differ significantly (*P<0.05*). [2]Beetles were dried for 24 h at 60 ^{o}C prior to weighing to ensure a uniform moisture concentration. Data were analyzed using a square-root transformation—actual means are reported.

TABLES

Table 1. Statistical tests[1] of the fixed effects for larval mortality in plant-based bioassays (2012).

Variable	Year	Site	Effect	d*f*	*t* value	*P* value
Mortality	2012	LaSalle	Treatment	7	3.672	0.0079
		Whiteside	Treatment	7	7.187	0.0002

[1] Statistical tests were performed using the Welch's *t* test, (α=0.05).

Table 2. Statistical tests[1] of the fixed effects of treatment for node-injury ratings, cumulative emergence, head capsule widths, and weights (2012-2013).

Variable	Year	Site	Effect	df_N	df_D	*F* value	*P* value
Node-injury rating	2012	LaSalle	Treatment	7	21.0	3.19	0.02
		Whiteside	Treatment	7	20.9	1.34	0.28
	2013	Champaign	Treatment	7	21.0	0.54	0.79
		LaSalle	Treatment	7	20.8	14.23	<0.0001
		Whiteside	Treatment	7	21.0	5.68	0.0009
Male emergence	2012	LaSalle	Treatment	7	21.0	2.39	0.05
		Whiteside	Treatment	7	21.0	6.00	0.0006
	2013	Champaign	Treatment	7	21.0	3.01	0.02
		Whiteside	Treatment	7	21.0	4.87	<0.01
Female emergence	2012	LaSalle	Treatment	7	21.0	2.01	0.10
		Whiteside	Treatment	7	21.0	1.88	0.12
	2013	Champaign	Treatment	7	21.0	1.46	0.24
		Whiteside	Treatment	7	21.0	3.95	<0.01
Male head capsule width	2012	LaSalle	Treatment	7	21.0	0.95	0.49
		Whiteside	Treatment	7	19.1	12.16	< 0.01
	2013	Champaign	Treatment	7	10.0	1.00	0.49
		Whiteside	Treatment	7	20.0	0.19	0.98
Female head capsule width	2012	LaSalle	Treatment	7	21.0	1.69	0.17
		Whiteside	Treatment	7	21.0	3.19	0.02
	2013	Champaign	Treatment	7	16.0	0.79	0.61
		Whiteside	Treatment	7	20.0	0.95	0.49
Male weight	2012	LaSalle	Treatment	7	21.0	0.53	0.81
		Whiteside	Treatment	7	19.3	1.45	0.24
	2013	Champaign	Treatment	6	9.0	1.36	0.32
		Whiteside	Treatment	7	20.0	1.51	0.22
Female weight	2012	LaSalle	Treatment	7	21.0	2.62	0.04
		Whiteside	Treatment	7	21.0	0.52	0.81
	2013	Champaign	Treatment	7	16.0	0.25	0.07
		Whiteside	Treatment	7	21.0	2.16	0.08

[1] Statistical tests were performed using PROC MIXED of SAS 9.3. The Kenward-Roger (1997) adjustment was used to approximate denominator degrees of freedom (df_D).